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THE MARINE BIOLOGICAL LABORATORY

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the Friday following the second Tuesday in August in each year at the Laboratory in Woods Hole, Massachusetts, at 9:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years, and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held promptly after the Annual Meeting of the Corporation at the Laboratory in Woods Hole, Mass. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years. After having served two consecutive terms of four years each, Trustees are ineligible for re-election until a year has elapsed. In addition, there shall be two groups of Trustees as follows:

(B) Trustees ex officio, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees Emeriti, who shall be elected from *present* or *former* Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees and who shall be elected for a term of five years and shall serve until his successor is selected and qualified; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees and who shall be selected for a term of five years and shall serve until his successor is selected and qualified; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. The Associates of the Marine Biological Laboratory shall be an unincorporated group of persons (including associations and corporations) interested in the Laboratory and shall be organized and operated under the general supervision and authority of the Trustees.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. REPORT OF THE DIRECTOR

To the Trustees of the Marine Biological Laboratory:

Gentlemen:

I submit herewith the report of the sixty-seventh session of the Laboratory.

The past year appears to have been quite satisfactory as regards the internal operation and scientific services of the Marine Biological Laboratory. The marine biological forms used more extensively by the investigators were abundant. There were few days when collections were impossible due to inclement weather. Two severe hurricanes did not seriously curtail the research and teaching programs as they came late in the season.

1. Hurricane Damage

Hurricane Carol hit Woods Hole on August 31 without adequate warning. The resulting high water flooded the basement of the Main Building to about the four-foot level. Through the heroic efforts of the staff and volunteers much valuable equipment was moved to floors above. However some of the heavy apparatus and equipment could not be moved, so suffered seriously in the flood waters. Also the building sustained damage, some of which may become apparent only in the future.

The Laboratory was most fortunate in a grant award of $50,000 by the National Science Foundation which will cover the major share of the uninsurable damage.

2. Plant Improvements

Continued progress has been made in the installation of heavier and additional electrical lines in the Main Building to carry the loads required by additional electronic equipment now used by the investigators.

A part of the grant from the National Science Foundation was used for some minor alterations in the buildings which, together with some standby precautions, should prevent a recurrence of damage in the buildings from flood waters of any future hurricanes.

A very pressing problem is the condition of the Crane Building. Built in 1918, this building has undergone serious and unavoidable deterioration in its interior walls, electrical wiring and plumbing. Also the building requires modernization for most effective use in the type of research currently under investigation at the Laboratory. Complete rehabilitation and modernization of this building will cost something of the order of $400,000.

3. Grants, Contracts and Contributions

Total income from these sources of support amounted to $97,840.19. This represents 21% of the total Laboratory budget.

Grants:

Rockefeller Foundation, general support	$20,000.00
National Science Foundation	10,000.00
American Philosophical Society	4,300.00
Frank R. Lillie Fellowship	1,500.00
American Cancer Society	6,600.00
National Institute of Health, studies of Arbacia egg and sperm cells	1,080.00
National Institute of Health, encephalization in *Ameiurus nebulosus* during ontogeny	2,012.00
New York Zoological Society	4,000.00

Contracts:

Office of Naval Research, studies in marine biology	15,000.00
Office of Naval Research, studies on marine populations and the effect of environmental factors	4,211.94
Office of Naval Research, function in nerve tissue using the giant axon of the squid	5,941.25
Atomic Energy Commission, studies in radiobiology on the biochemistry of cell nuclei	12,460.00
Atomic Energy Commission, radiobiological studies on the physiology of marine organisms	6,590.00

Contributions:

The annual contribution from the M. B. L. Associates was $3,020, almost double that of the preceding year. This is very encouraging and it is hoped that future contributions will continue to increase. Special contributions were made by Mrs. Edward B. Meigs, Dr. Winterton C. Curtis and Dr. William D. Curtis. Dr. Matilda M. Brooks contributed $2,000 toward the new addition of the M. B. L. Club House in memory of her husband, Dr. Sumner C. Brooks, who was associated for many years with the Laboratory as a member of the staff in the course in Marine Botany, as an investigator, and as a Trustee.

4. Instruction

This past year Dr. Harold Bold served very effectively as head of the course in Marine Botany and has consented to continue in that post. Dr. Lewis H. Kleinholz is retiring as head of the course in Invertebrate Zoology having completed a very successful five-year term in this position. He will be succeeded by Dr. Theodore H. Bullock who has spent some summers at the Laboratory as an independent investigator.

5. Bar Neck Property

Negotiations on the Bar Neck Property have progressed satisfactorily during the past year and we may reasonably expect that a final settlement of this problem will be achieved in the spring of 1955. The final price which has been tentatively agreed on appears equitable as compared with an appraised value determined for the Laboratory.

6. Fellowships and Scholarships

The Laboratory was fortunate in the establishment of three fellowships or scholarships by friends of the Laboratory. These are described in the Annual Announcement. Mrs. Gary N. Calkins established one in memory of her husband, Dr. Gary N. Calkins. Mrs. Kimball Chase Atwood III established one in memory of her mother, Emma Coote Drew and friends and former students of Dr. Edwin Grant Conklin established a scholarship in his memory.

7. Changes in the By-Laws

Members of the Corporation should note certain changes in the By-Laws which are included in this Annual Report. These changes appear in Article III, changing the date and time of the Annual Meeting of the Corporation, Article VII, changing the date and time of the Annual Meeting of the Trustees, and Article X, clarifying the relation of the M. B. L. Associates to the Laboratory.

Respectfully submitted,

PHILIP B. ARMSTRONG,
Director

1. THE STAFF, 1954

PHILIP B. ARMSTRONG, Director, State University of New York, School of Medicine, Syracuse

SENIOR STAFF OF INVESTIGATION

A. P. MATHEWS, Professor of Biochemistry, *Emeritus,* University of Cincinnati
G. H. PARKER, Professor of Zoology, *Emeritus,* Harvard University

ZOOLOGY

I. CONSULTANTS

F. A. BROWN, JR., Professor of Zoology, Northwestern University
LIBBIE H. HYMAN, American Museum of Natural History
A. C. REDFIELD, Woods Hole Oceanographic Institution

II. INSTRUCTORS

L. H. KLEINHOLZ, Professor of Biology, Reed College, in charge of course
JOHN H. LOCHHEAD, Associate Professor of Zoology, University of Vermont
NORMAN A. MEINKOTH, Associate Professor of Zoology, Swarthmore College
GROVER STEPHENS, Assistant Professor of Zoology, University of Minnesota
JOHN M. ANDERSON, Associate Professor of Zoology, Cornell University
MURIEL SANDEEN, Department of Zoology, Duke University
L. M. PASSANO, Department of Zoology, University of Washington, Seattle
MORRIS ROCKSTEIN, Department of Physiology, New York University, Bellevue Medical Center

III. LABORATORY ASSISTANTS

WILLIAM E. DOSSEL, Johns Hopkins University
DOROTHY M. SKINNER, Tufts College

EMBRYOLOGY

I. Instructors

S. Meryl Rose, Associate Professor of Zoology, University of Illinois, in charge of course
William E. Berg, Assistant Professor of Zoology, University of California, Berkeley
Mac V. Edds, Jr., Associate Professor of Biology, Brown University
John R. Shaver, Assistant Professor of Zoology, University of Missouri
J. P. Trinkaus, Assistant Professor of Zoology, Yale University
Edgar Zwilling, Associate Professor of Genetics, University of Connecticut

II. Laboratory Assistants

Joan K. Ericksen, Radcliffe College
Roger D. Milkman, Harvard University

PHYSIOLOGY

I. Consultants

Merkel H. Jacobs, Professor of Physiology, University of Pennsylvania
Otto Loewi, Professor of Pharmacology, New York University, School of Medicine
Arthur K. Parpart, Professor of Biology, Princeton University
Albert Szent-Gyorgyi, Director, Institute for Muscle Research, Woods Hole
E. S. Guzman Barron, Associate Professor of Biochemistry, University of Chicago

II. Instructors

Daniel Mazia, Associate Professor of Zoology, University of California, in charge of course
Stephen Kuffler, Associate Professor of Ophthalmology, Wilmer Institute, Johns Hopkins University Medical School
Max A. Lauffer, Professor and Head of Dept. of Biophysics, University of Pittsburgh
George Wald, Professor of Biology, Harvard University
Andrew Szent-Gyorgyi, Independent Investigator, The Institute for Muscle Research
James D. Watson, Senior Research Fellow, California Institute of Technology

III. Laboratory Assistant

Paul Bernstein, Department of Zoology, Columbia University

BOTANY

I. Consultants

Maxwell S. Doty, Associate Professor of Botany, University of Hawaii
Wm. Randolph Taylor, Professor of Botany, University of Michigan

II. Instructors

Harold C. Bold, Professor of Biology, Vanderbilt University, in charge of course
Franz Moewus, Privatdozent, University of Heidelberg and Research Associate, Department of Zoology, Columbia University
Richard C. Starr, Instructor in Botany, University of Indiana

III. Laboratory Assistants

Walter Herndon, Vanderbilt University

IV. Collector

Ann Allen, University of Indiana

MARINE ECOLOGY

I. Consultants

W. C. Allee, University of Florida
Alfred C. Redfield, Woods Hole Oceanographic Institution

II. Instructors

Bostwick H. Ketchum, Marine Microbiologist, Woods Hole Oceanographic Institution, in charge of course
Edwin T. Moul, Assistant Professor of Botany, Rutgers University
Charles Jenner, Assistant Professor of Zoology, University of North Carolina

III. Assistant

Edward J. Kuenzler, Department of Biology, University of Georgia

THE LABORATORY STAFF, 1954

Homer P. Smith, General Manager

Mrs. Deborah Lawrence Harlow, Librarian
James McInnis, Manager of Supply Department
Robert Kahler, Superintendent, Buildings and Grounds
Robert B. Mills, Manager, Department of Research Service

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Polly L. Crowell
Mrs. Lila Myers
Nancy Shave
Elizabeth Correllus

LIBRARY

Mary E. Castellano, Assistant Librarian

Mary A. Rohan
Naomi Botelhe
Albert Neal

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Arthur Callahan
Robert Gunning
John Head
George A. Kahler
Donald B. Lehy
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James S. Thayer

DEPARTMENT OF RESEARCH SERVICE

GAIL M. CAVANAUGH SEAVER HARLOW
PATRICIA PHILPOTT

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MILTON B. GRAY
WALTER E. KAHLER
ROBERT PERRY
PATRICIA M. CONWAY
GEOFFREY LEHY
ROBERT O. LEHY
CARL SCHWEIDENBACK
JAMES P. WHITCOMB
H. S. WAGSTAFF

BIOLOGICAL BULLETIN

DONALD P. COSTELLO, Managing Editor
University of North Carolina, Dept. of Zoology
Chapel Hill, North Carolina
CATHERINE HENLEY, Assistant to the Editor

2. INVESTIGATORS AND STUDENTS

Independent Investigators, 1954

ALLEE, W. C., Head, Department of Biology, University of Florida
ALLEN, M. JEAN, Assistant Professor of Zoology, University of New Hampshire
AMBERSON, WILLIAM R., Professor of Physiology, University of Maryland Medical School
AMES, BRUCE N., Research Fellow, National Institute of Health
ANDERSON, JOHN M., Associate Professor of Zoology, Cornell University
ARMSTRONG, JOHN C., 1165 5th Avenue, New York City, New York
ARMSTRONG, PHILIP B., Professor of Anatomy, State University of New York, College of Medicine at Syracuse
BANG, FREDERIK, Professor of Parasitology, Johns Hopkins University School of Hygiene
BARTLETT, JAMES H., Professor of Physics, University of Illinois
BARTON, JAY II, Assistant Professor of Zoology, Columbia University
BERGER, CHARLES A., Chairman, Biology Department, Fordham University
BLOCH, KONRAD, Professor of Biochemistry, Harvard University
BLUM, HAROLD F., Physiologist and Visiting Lecturer, Princeton University
BOETTIGER, EDWARD G., Associate Professor of Zoology, University of Connecticut
BOLD, HAROLD C., Professor of Biology, Vanderbilt University
BROWN, FRANK A., JR., Professor and Chairman, Department of Biological Sciences, Northwestern University
BUTLER, ELMER G., Professor of Biology, Princeton University
CABIB, ENRICO, Visiting Fellow, Columbia University
CANTONI, G. L., Associate Professor in Pharmacology, Western Reserve University
CARROLL, FLORA, Independent Investigator, The Institute for Muscle Research
CHANG, JOSEPH J., Research Fellow, Princeton University
CHASE, AURIN M., Associate Professor of Biology, Princeton University
CHENEY, RALPH HOLT, Professor of Biology, Brooklyn College
CLARK, ELIOT R., Professor *Emeritus* of Anatomy, The Wistar Institute, University of Pennsylvania
CLEMENT, A. C., Associate Professor of Biology, Emory University
CLOWES, G. H. A., Research Director *Emeritus*, Eli Lilly and Company
COHEN, ADOLPH I., Research Fellow, University of California
COLE, KENNETH S., Technical Director, Naval Medical Research Institute
COLLIER, JACK R., Graduate Student, University of North Carolina

COOPERSTEIN, SHERWIN J., Assistant Professor of Anatomy, Western Reserve University School of Medicine
CORNMAN, IVOR, Assistant Research Professor in Anatomy, George Washington University
COSTELLO, DONALD P., Kenan Professor of Zoology and Chairman, University of North Carolina
DIETER, C. D., Professor of Biology, Director of Pediatric Research, Michael Reese Hospital
DWYER, JOHN D., Director of Department of Biology, Saint Louis University
EDDS, MAC V., JR., Associate Professor of Biology, Brown University
ELLIOTT, ALFRED M., Associate Professor of Zoology, University of Michigan
EPHRUSSI, BORIS, Professor at the Faculté des Sciences, Paris
EYZAGUIRRE, CARLOS, Guggenheim Fellow, Wilmer Institute, Johns Hopkins Hospital
FAILLA, G., Professor of Radiology, Columbia University
FITZHUGH, RICHARD, Instructor, Wilmer Institute, Johns Hopkins Hospital
FRAENKEL, G. S., Professor of Entomology, University of Illinois
FRIES, E. F. B., Associate Professor, The City College of New York
GALL, JOSEPH G., Instructor in Zoology, University of Minnesota
GASTEIGER, EDGAR L., Associate in Physiology, Harvard Medical School
GERSH, ISIDORE, Professor of Anatomy, The University of Chicago
GOODCHILD, C. G. Associate Professor of Biology, Emery University, Georgia
GRANT, PHILIP, Research Associate, The Institute for Cancer Research
GRELL, KARL, Professor of Zoology, Max Planck Inst. f. Biologie, Tuebingen, Western Germany
GROSCH, DANIEL S., Associate Professor of Genetics, North Carolina State College
GRUNDFEST, HARRY, Associate Professor of Neurology, College of Physicians and Surgeons
GUTTMAN, RITA, Assistant Professor, Brooklyn College
HAMBURGH, MAX, Research Fellow and Instructor in Anatomy, University of Pennsylvania
HARVEY, ETHEL BROWNE, Research Investigator in Biology, Princeton University
HARVEY, E. NEWTON, Professor of Physiology, Princeton University
HAYASHI, TERU, Associate Professor of Zoology, Columbia University
HAYWOOD, CHARLOTTE, Professor of Physiology, Mount Holyoke College
HEILBRUNN, L. V., Professor of Zoology, University of Pennsylvania
HEMPLING, HAROLD G., Instructor of Physiology, University of Pennsylvania School of Medicine
HENLEY, CATHERINE, Research Associate, University of North Carolina
HERVEY, JOHN P., Electronic Engineer, Rockefeller Institute for Medical Research
HOFFMAN, JOSEPH F., Lecturer and Research Associate, Princeton University
HOLTZER, HOWARD, Associate in Anatomy, University of Pennsylvania School of Medicine
HURLBUT, WILLIAM PAUL, Assistant, Johns Hopkins University
HUNT, CARLTON C., Associate, Rockefeller Institute for Medical Research
HUTTER, OTTO, Rockefeller Fellow from England, Wilmer Institute, Johns Hopkins Hospital
JACOBS, M. H., Professor of General Physiology, University of Pennsylvania Medical School
JENKINS, GEORGE B., *Emeritus* Professor of Anatomy, George Washington University
JENNER, CHARLES E., Associate Professor of Zoology, University of North Carolina
JOHNSON, FRANK H., Associate Professor of Biology, Princeton University
JOHNSON, WILLIAM HARDING, Instructor in Physiology, University of Illinois
KARREMAN, GEORGE, Research Associate, Institute for Muscle Research
KAUFMAN, SEYMOUR, National Institute of Health
KEMPTON, RUDOLF T., Professor and Chairman, Department of Zoology, Vassar College
KEOSIAN, JOHN, Professor of Biology, Rutgers University
KING, THOMAS J., Research Associate, Institute for Cancer Research
KLEINHOLZ, L. H., Professor of Biology, Reed College
KLOTZ, IRVING M., Professor of Chemistry and Biology, Northwestern University
KOCH, HENRI, Advanced Fellow of the Belgian American Educational Foundation
KRAHL, MAURICE E., Professor of Physiology, University of Chicago
KUFFLER, STEPHEN W., Instructor, Wilmer Institute, Johns Hopkins Hospital
KUNTZ, ELOISE, Assistant Professor of Physiology, Vassar College
LANSING, ALBERT I., Associate Professor in Anatomy, Washington University Medical School
LAUFFER, MAX A., Head, Department of Biophysics, University of Pittsburgh
LEDERBERG, JOSHUA, Associate Professor of Genetics, University of Wisconsin

LEVY, MILTON, Professor of Chemistry, New York University, Bellevue Medical Center
LIPKE, HERBERT, Research Associate, University of Illinois
LITT, MORTIMER, Research Fellow in Bacteriology, Harvard Medical School
LOCHHEAD, JOHN H., Professor of Zoology, University of Vermont
LOEWENSTEIN, WERNER, Kellogg Fellow from Chile, Wilmer Institute, Johns Hopkins Hospital
LORAND, LASZLO, Assistant Professor, Wayne University College of Medicine
LORAND, JOYCE A., 16834 Evanston, Detroit 24, Michigan
LOVE, WARNER E., Fellow, Johnson Foundation, University of Pennsylvania
MARSLAND, DOUGLAS, Professor of Biology, Washington Square College
MOEWUS, FRANZ, Columbia University
MCLAUGHLIN, JANE, Independent Investigator, The Institute for Muscle Research
MEINKOTH, NORMAN A., Associate Professor of Biology, Swarthmore College
MENKIN, VALY, Head of Experimental Pathology, Temple University School of Medicine
METZ, CHARLES B., Associate Professor of Zoology, Florida State University
MIDDLEBROOK, ROBERT, Institute for Muscle Research
MONROE, CLARENCE L. E., Professor of Biology, Morgan State College
MOORE, GEORGE M., Professor of Zoology and Chairman of Zoology Department, University of New Hampshire
MOORE, JOHN W., Biophysicist, Naval Medical Research Institute
MOTOMURA, ISAO, Professor Biological Institute of Tohoku University, Sendai, Japan
MOUL, EDWIN T., Assistant Professor of Botany, Rutgers University
NACHMANSOHN, DAVID, Associate Professor of Neurology, College of Physicians & Surgeons
NANNINGA, LUDO, Independent Investigator, The Institute for Muscle Research
NARDONE, ROLAND, Assistant Professor of Biology, Catholic University of America
NELSON, LEONARD, Assistant Professor of Physiology, University of Nebraska
OSTERHOUT, W. J. V., Member *Emeritus*, Rockefeller Institute for Medical Research
PARPART, ARTHUR K., Chairman, Department of Biology, Princeton University
PASSANO, LEONARD M., Instructor of Zoology, University of Washington
PERSON, PHILIP, CAPT., Dental Corps, Walter Reed Army Medical Center
PETTIBONE, MARIAN H., Assistant Professor of Zoology, University of New Hampshire
PHILPOTT, DELBERT, Independent Investigator, The Institute for Muscle Research
PICK, JOSEPH, Associate Professor of Anatomy, New York University College of Medicine
PIERCE, MADELENE E., Professor of Zoology, Vassar College
PROCTOR, NATHANIEL K., Associate Professor, Morgan State College
RAY, CHARLES, JR., Assistant Professor of Biology, Emory University
RAY, DAVID T., Instructor of Zoology, Howard University
RIESER, PETER, Research Associate, University of Pennsylvania
ROCKSTEIN, MORRIS, Assistant Professor of Physiology, New York University, Bellevue Medical Center
RONKIN, R. R., Associate Professor of Biological Sciences, University of Delaware
ROSE, S. MERYL, Professor of Zoology, University of Illinois
RUGH, ROBERTS, Associate Professor of Radiology, Columbia University
SAGER, RUTH, Assistant, Rockefeller Institute for Medical Research
SANDEEN, MURIEL I., Instructor in Zoology, Duke University
SCHARRER, ERNST, Associate Professor of Anatomy, University of Colorado, School of Medicine
SCHARRER, BERTA, Assistant Professor, University of Colorado, School of Medicine
SCHECHTER, VICTOR, Associate Professor of Biology, City College of New York
SCHIFFMAN, GERALD, Research Associate, Institute for Muscle Research
SCLUFER, EVELYN, National Science Foundation
SCOTT, ALLAN, Professor of Biology, Chairman of the Department, Colby College
SCOTT, DWIGHT B. MCNAIR, Assistant Professor, Robinson Foundation, University Hospital
SCOTT, SISTER FLORENCE MARIE, Professor of Biology, Seton Hill College
SCOTT, GEORGE T., Professor of Zoology, Oberlin College
SHANES, ABRAHAM M., Investigator in Animal Physiology, National Institute of Health
SHAVER, JOHN R., Assistant Professor of Zoology, University of Missouri
SLIFER, ELEANOR H., Associate Professor of Zoology, State University of Iowa
SMITH, A. H. REESOR, Electronics Engineer, Rockefeller Institute for Muscle Research

SONNENBLICK, B. P., Associate Professor of Biology, Rutgers University
SPEIDEL, CARL C., Professor of Anatomy and Chairman of Department, University of Virginia, School of Medicine
SPIEGEL, MELVIN, Research Fellow, California Institute of Technology
STARR, RICHARD, Assistant Professor of Botany, Indiana University
STEELE, RICHARD H., Instructor of Biochemistry, Tulane University
STEINBERG, MALCOLM S., Graduate Student, University of Minnesota
STEPHENS, GROVER C., Instructor in Zoology, University of Minnesota
STOKEY, ALMA G., Professor *Emeritus* of Plant Science, Mount Holyoke College
STREHLER, BERNARD L., Assistant Professor, University of Chicago, Institute of Radiobiology and Biophysics
STUNKARD, HORACE W., Professor of Biology, New York University
STURTEVANT, ALFRED H., Professor of Biology, California Institute of Technology
SWIFT, HEWSON H., Assistant Professor in Zoology, University of Chicago
SZENT-GYORGYI, ANDREW G., Independent Investigator, Institute for Muscle Research
SZENT-GYORGYI, ALBERT, Director of Research, Institute for Muscle Research
SZENT-GYORGYI, EVA, Independent Investigator, Institute for Muscle Research
TRACY, HENRY C., Associate in Anatomy, University of Mississippi
TRAGER, WILLIAM, Associate Member, Rockefeller Institute
TRINKAUS, J. P., Assistant Professor of Zoology, Osborn Zoological Laboratory, Yale University
TROLL, WALTER, Assistant Director and Assistant Professor in Biochemistry, May Institute
TSUJI, FREDERICK I., Princeton University
TYLER, ALBERT, Professor of Embryology, California Institute of Technology
DEVILLAFRANCA, GEORGE W., Research Associate, Institute for Muscle Research
VILLEE, CLAUDE A., Assistant Professor of Biochemistry, Harvard Medical School
VINCENT, WALTER S., Instructor in Anatomy, State University of New York, School of Medicine
WARNER, ROBERT C., Assistant Professor of Chemistry, New York University, Bellevue Medical Center
WEBB, H. MARGUERITE, Research Associate, Goucher College
WELLS, G. P., Reader in Zoology, University College, London
WHITING, P. W., Professor of Zoology *Emeritus*, University of Pennsylvania
WHITING, ANNA R., Lecturer in Zoology, University of Pennsylvania
WHITTAKER, VICTOR PERCY, Assistant Professor of Physiology, University of Cincinnati, College of Medicine
WICHTERMAN, RALPH, Professor of Biology, Temple University
WILCZYNSKI, JAN, Professor of Biology and Genetics, State Lebanese University, Beirut
WILSON, WALTER L., Assistant Professor of Physiology and Biophysics, University of Vermont College of Medicine
WRIGHT, PAUL A., Assistant Professor of Zoology, University of Michigan
ZWILLING, EDGAR, Associate Professor, University of Connecticut
ZWEIFACH, BENJAMIN W., Associate Professor of Biology, New York University, Washington Square College

3. LALOR FELLOWS, 1954

AMES, BRUCE, National Institutes of Health
COLLIER, JACK R., University of North Carolina
EPHRUSSI, BORIS, Faculté des Sciences, Paris
EPHRUSSI, HARRIET TAYLOR, Faculté des Sciences, Paris
GALL, JOSEPH, University of Minnesota
STREHLER, B. L., University of Chicago
VINCENT, WALTER, State University of New York, School of Medicine at Syracuse
JOHNSON, WILLIAM HARDING, University of Illinois

Beginning Investigators, 1954

BARR, H. JAY, University of Pennsylvania
BERNSTEIN, GERALD S., Lalor Predoctoral Research Fellow, University of Delaware
BORYSKO, EMIL, Damon Runyon Memorial Fund Research Fellow, Johns Hopkins University
BUHITE, ROBERT JOHN, Graduate Fellow, Saint Louis University
FLEISCHER, MARILYN, Teaching Fellow, New York University
FRIEDMAN, STANLEY, Research Associate in Entomology, University of Illinois
FUSCO, MADELINE M., Instructor, Vassar College
GROSOF, JESSICA, Student, Wellesley College
HANSEN, D. D., Grass Trust Fellow, University of California School of Medicine
HAUBRICH, ROBERT, Graduate Assistant, University of Florida
KROJANKER, IRENE, University of Pennsylvania
LANDAU, JOSEPH V., Damon Runyon Cancer Research Fellow, New York University
MOOS, CARL, Graduate Student, Columbia University
PEPPER, MAX, Student, University of Pennsylvania School of Medicine
POTTER, DAVID D., Graduate Student, Harvard University
SMALL, JEAN E., Graduate Assistant, Brown University
TUNIK, BERNARD D., Graduate Student, Columbia University
VENNESLAND, BIRGIT, University of Chicago
WARREN, MARTHA M., Teaching Assistant in Biology, Brown University
WERNTZ, HENRY OSCAR, Graduate Student, Yale University

Research Assistants, 1954

ALLEN, M. ANN, Indiana University
BARNES, LOY J., University of Missouri
BENNETT, MIRIAM F., Northwestern University
CAHN, ROBERT DAVID, Swarthmore College
CHALFIN, DAVID, Princeton University
DOSSEL, WILLIAM E., Johns Hopkins University
DRAKE, JOHN W., Yale University
ERDMAN, HOWARD E., Lehigh University
ERICKSON, JOAN, Radcliffe College
FROST, JAMES L., Johns Hopkins University School of Medicine
GERGEN, JOHN A., Harvard Medical School
GAEUMAN, JOHN, Oberlin College
GROSS, MILTON M., State University of New York, College of Medicine at New York City
GROVES, PEGGY WOFORD, Yale University
GRUNDFEST, BROOKE, Columbia University
HAYWARD, HUGH R., University of Rochester
HIGGINS, DON CHENEY, State University of New York, College of Medicine at Syracuse
ISENBERG, IRVIN, University of Chicago
KRUPA, PAUL L., New York University
LACHANCE, LEO E., North Carolina State College
LARIS, PHILIP C., Princeton University
LEVEQUE, THEODORE F., University of Colorado School of Medicine
LORING, JANET MASON, Harvard Medical School
MAWE, RICHARD C., Princeton University
MESELSON, MATT S., California Institute of Technology
MICHAELSON, I. ARTHUR, University of Cincinnati College of Medicine
MILKMAN, ROGER D., Harvard University
PAUL, ANDREW
RALPH, CHARLES L., Northwestern University
SHANKLIN, DOUGLAS R., State University of New York College of Medicine at Syracuse
SHEDLOVSKY, ALEXANDRIA, Rockefeller Institute for Medical Research
SHELBURNE, JAMES CHRISTIE, Yale University
SKINNER, DOROTHY M., Tufts College

STROUD, AGNES N., Argonne National Laboratories
SUBTELNY, STEPHEN S., University of Missouri
SULLIVAN, ROBERT LITTLE, North Carolina State College
SZENT-GYORGYI, MARTA, Institute for Muscle Research
VOORHEES, DAVID B., Yale University
WELLINGTON, FREDERICA MERCER, Harvard Medical School
ZIMMERMAN, ARTHUR M., New York University

Library Readers, 1954

BALL, ERIC G., Professor of Biological Chemistry, Harvard Medical School
BELOFF, RUTH H., Graduate Student, Yale University
BODANSKY, OSCAR, Professor of Biochemistry, Cornell Medical College
BURWELL, E. LANGDON, Falmouth Medical Center
COHEN, SEYMOUR S., Associate Professor of Physiological Chemistry and Pediatrics, University of Pennsylvania
DIXON, FRANK J., Chairman and Professor of Pathology, University of Pittsburgh
DORFMAN, ALBERT, Associate Professor of Pediatrics, University of Chicago
DUBOIS, EUGENE F., *Emeritus* Professor of Physiology, Cornell University Medical College
EDEN, MURRAY, Special Fellow, Princeton University
EICHEL, HERBERT J., Research Associate, Hahnemann Medical College
EISENBERG, MAX A., Research Associate, Columbia Universtiy
FITTING, CHARLOTTE, Research Fellow in Bacteriology, Harvard Medical School
FREUND, JULES, Chief of Division of Applied Immunology, Public Health Research Institute of the City of New York
FRIAUF, JAMES J., Associate Professor of Biology, Vanderbilt University
GABRIEL, MORDECAI L., Assistant Professor of Biology, Brooklyn College
GATES, RUGGLES, *Emeritus* Professor, University of London, at Harvard University
GINSBERG, HAROLD S., Associate Professor of Preventive Medicine, Western Reserve University, School of Medicine
GOTTSCHALL, GERTRUDE, Associate, College of Physicians and Surgeons
GREIF, ROGER L., Assistant Professor of Physiology, Cornell University Medical College
GUDERNATSCH, FREDRICK, Cornell University Medical College
GUREWICH, VLADIMAR, 333 Central Park West, New York 25
HAMMOND, WARNER S., Associate Professor of Anatomy, State University of New York, Upstate Medical Center
JONES, SARAH R., Instructor in Zoology, Connecticut College
KABAT, ELVIN A., Professor of Microbiology, College of Physicians and Surgeons
KARUSH, FRED, Associate Professor of Immunology, University of Pennsylvania School of Medicine
KIMBALL, CHARLES P., West Barnstable, Mass.
KRAMER, MOLLIE P., Bibliographer, American Meteorological Society
LEVINE, RACHMIEL, Chairman, Department of Medicine, Michael Reese Hospital
LEVY, ARTHUR L., Research Biochemist, St. Vincent's Hospital
LING, GILBERT, Assistant Professor of Neurophysiology, University of Illinois, Chicago
LOEWI, OTTO, Research Professor, New York University College of Medicine
LOVE, LOIS H., National Research Council
LOWENSTEIN, OTTO, Research Associate, College of Physicians and Surgeons
MCDONALD, SISTER ELIZABETH SETON, Chairman Biology Department, College of Mt. St. Joseph on the Ohio
MESELSON, MATT S., Graduate Student, California Institute of Technology
PLOUGH, H. H., Amherst College
RACKER, EFRAIM, Chief, Division of Nutrition and Physiology, Public Health Research Institute
RATNOFF, OSCAR D., Assistant Professor of Medicine, Western Reserve University School of Medicine

ROOT, WALTER S., Professor of Physiology, College of Physicians and Surgeons
RUBIN, SAUL H., Director of Applied Research, Hoffmann-LaRoche Inc.
SHAHN, EZRA, Assistant, Sloan Kettering Institute
SHEDLOVSKY, THEODORE, Associate Member, Rockefeller Institute
SONNEBORN, T. M. Professor of Zoology, Indiana University
STEINHARDT, JACINTO, Director Operations Evaluation Group, Navy Department
SULKIN, S. EDWARD, Professor and Chairman, Department of Microbiology, Southwestern Medical School
TAUBER, HANS-ZUKAS, Assistant Professor of Neurology, New York University College of Medicine
ULRICH, FRANK, Research Fellow in Physiology, Yale University School of Medicine
VISHNIAC, WOLF, Assistant Professor of Microbiology, Yale University
WAINIO, WALTER W., Associate Professor of Biochemistry, Rutgers University
WEBER, ANNEMARIE, Research Associate, College of Physicians and Surgeons
WILSON, IRWIN B., Assistant Professor of Biochemistry, College of Physicians and Surgeons
WOLFF, WALTER H., Principal, Wm. C. Bryant High School

Students, 1954

BOTANY

APOLLONIO, SPENCER, Bowdoin College
CORWIN, ELIZABETH GAYER, Ohio University
DAVIS, ROBERTA L., Clark University
FISHER, MARY A., Drew University
LAETSCH, WATSON M., Wabash College
LARSON, JANE L., Clark University
MACFADGEN, JOSEPHINE HELEN, Acadia University
MASIELLO, MARIANNA, Hunter College
PASTORIUS, JANICE I., Mount Holyoke College
REDDING, WILLIAM F., DePaul University
TRAINOR, FRANCIS R., Vanderbilt University
WOODSON, JOANNA, Woman's College, University North Carolina

EMBRYOLOGY

BAKER, K. FRANCE, Columbia University
BRAUNWALD, JACK, Amherst College
BRONSWEIG, RUTH D., University of Florida
BUHITE, ROBERT J., Saint Louis University
DECKER, JOHN D., Hartwick College
DEFANDORF, VIRGINIA, Wesleyan University
DOWLING, JOHN A., Harvard College
FLEISCHMAN, JULIAN BURD, Yale University
FRY, DOROTHY S., University of Missouri
GIACINO, ROSARIA MARIA, Mount Holyoke College
GROSOF, JESSICA, Wellesley College
HARRIS, JOSEPH DAVID, Purdue University
IZEMAN, HENRY FRANK, Brown University
KALLAPRAVIT, BOONANAKE, Washington University
KAVEE, ROBERT C., University of Rochester
KNOWLES, ROBERT C., Brown University
KONING, ALICE LOUISE, Iowa State College
MCLEOD, GUY COLLINGWOOD, Waquoit, Massachusetts
MARKO, ANITA R., Adelphi College
MOMBERG, HAROLD LESLIE, Missouri University

NALL, THOMAS MARTIN, Wesleyan University
PETERSEN, JEAN HELEN, Mount Holyoke College
ROBERTS, JANE CAROLYN, University of Massachusetts
RONDON-TARCHETTI, TERESA, Yale University
SHEPARD, JAMES EDWARD, Wesleyan University
SZYMANSKI, CHRISTINE DOLORES, Pennsylvania College for Women
THURMAN, SUSAN EMILY, Bryn Mawr
VALERIAN, SISTER M., Marquette University
WIDMAYER, DOROTHEA JANE, Wellesley College

PHYSIOLOGY

AMBELLAN, ELISABETH H., Columbia University
BORENFREUND, ELLEN, New York University
BROOKES, VICTOR J., University of Illinois
BURGOS, MARIO H., Harvard Medical School
DEFLORIDA, FRANCISCO A., Tufts College
DONOHOO, JOHN T., Notre Dame University
EDWARDS, JOSHUA LEROY, Rockefeller Institute for Medical Research
FROST, JAMES L., Johns Hopkins University School of Medicine
FULLER, GEORGE RIPLEY, Cornell Medical College
HILL, ROBERT BENJAMIN, Harvard University
HONG, SUK KI, University of Rochester
IODICE, ARTHUR ALFONSO, Syracuse University
ISAACSON, ALLEN, Harvard University
KADIS, SOLOMON, Vanderbilt University
KANE, ROBERT EDWARD, Johns Hopkins University
KELLY, ARTHUR LOUIS, Scripps Institution
LE ROY, ANDREE, University of Montreal
LEVY, CHARLES KINGSLEY, University of North Carolina
LITTAU, VIRGINIA CONWAY, Columbia University
MAURIELLO, GEORGE E., New York University
RABINOVITCH, MICHEL P., Medical School, University of S. Paulo, Brazil
SANTOS, PERSIO DE SOUZA, University of Pittsburgh
SANTOS, HELEN A. L., University of Pittsburgh
STAHL, FRANKLIN WILLIAM, University of Rochester
VILLAVICENCIO, MARINO, University of Chicago
WARD, JOHN M., Rutgers University
WARGO, J. DONALD, University of Minnesota
ZATZMAN, MARVIN LEON, Ohio State University

INVERTEBRATE ZOOLOGY

ALBRIGHT, RAYMOND GERARD, Loyola University
AMENTA, PETER SEBASTIAN, Marquette University
ANDRUS, WILLIAM DEWITT, JR., Oberlin College
ARAB, YOOSIF MOHAMMED, Johns Hopkins University
ATKIN, ADAM, Antioch College
BALBINDER, ELIAS, Indiana University
BARKI, GERDA S., University of California
BARNETT, AUDREY JANE, Wilson College
BASCH, PAUL FREDERICK, City College of New York
BAUDER, LOIS ELIZABETH, Hiram College
BELDEN, DON A., JR., Washington State College
BENDER, MARILYN IRIS, Hunter College

BLAISDELL, MARJORIE RUTH, Middlebury College
BOTKIN, DOROTHY ANN, University of Rochester
BROWN, ROBERT ALEXANDER, Harvard College
BURNETT, ALLISON LEE, Cornell University
CHANG, PATRICIA C. H., Johns Hopkins University
CHILD, FRANK MALCOLM, III, University of California
COHEN, MATANAH, Forest Hills 75, New York
COOLEY, LOUISE M., University of Massachusetts
DAVIS, ROWLAND HALLOWELL, Harvard University
DECK, LUCIUS LINTON, Ford Foundation Grant
EVANS, DAVID ROBERT, George Washington University
FRIAUF, JAMES J., Vanderbilt University
GENEST, ALLEGRA A., Tufts (Jackson College for Women)
GINSBERG, ELLEN G., Swarthmore College
GOOLSBY, CHARLES M., Harvard University
GOTTLIEB, SELMA G., University of Pennsylvania
HEIMAN, EMMY LOU, Oberlin College
HOGAN, MARGARET ROSE, Saint Louis University
HUTTON, KENNETH EARL, Purdue University
JOHN, MARY C., Fordham University
JOSEPH, P. V., Fordham University
KAOCHARERN, PANEE, University of Wisconsin
KAYE, GORDON ISRAEL, Columbia University
KERR, JOHN P., Rutgers University
KERR, NORMAN STORY, Oberlin College
LESCURE, ONO L., Ruxton, Maryland
LITTMAN, DAVID BENJAMIN, Brooklyn College
MARKER, MURIEL JOSEPHINE, Bard College
MCCANN, FRANCES VERONICA, University of Illinois
MCMILLAN, ROSAMOND, Northwestern University
NAWAR, GERGIS, Washington University
OTERO, LUIS RAUL, George Washington University
PETERS, LEWIS E., JR., Purdue University
QUINN, SISTER MARY LORITA, University of Notre Dame
RESNER, RAYMOND, Long Island University
RICHARDS, CHRISTINA M., University of Illinois
ROHS, ROBERT RYAN, Massachusetts Institute of Technology
RYAN, BROTHER SIMEON, Saint Louis University
SAGE, JANET K., DePauw University
DETERRA, NOEL, Barnard College
TOMLIN, PATRICIA ANN, Mount Holyoke College
DELVECCHIO, ROBERT JAMES, Fordham Graduate School
VOGEL, JHILIP H., S.J., West Baden College
YOUNGPETER, JOHN MICHAEL, Pontiac, Michigan, Senior High School

ECOLOGY

CROWELL, KENNETH LELAND, Yale University
EIKE, ROBERTA CLAIRE, Elmira College
GOTTLIEB, MEYER L., 1801 Loring Place, Bronx
HOFFMAN, ROBERT SHAW, University of California
MILLER, WILLIAM T., Government of the Virgin Islands
ROBINSON, MARIUS, Stanford University
SMITH, ANN HASWELL, Wesleyan University
VOORHEES, DAVID BRUCE, Yale University
WOLFE, MARGARET K., Harvard University

4. Tabular View of Attendance, 1950–1954

	1950	*1951*	*1952*	*1953*	*1954*
Investigators—Total	338	303	306	310	298
Independent	198	186	172	176	180
Under Instruction	43	28	38	37	20
Library Readers	48	37	49	46	52
Research Assistants	49	52	47	51	46
Students—Total	126	124	123	136	134
Zoology	55	55	55	55	56
Embryology	29	27	23	30	29
Physiology	27	29	27	31	28
Botany	13	13	11	11	12
Ecology			7	9	9
Total Attendance	464	427	429	446	432
Less persons registered as both students and investigators			2		5
	464	427	427	446	427
Institutions Represented—Total	156	158	149	155	136
By Investigators	114	115	92	90	104
By Students	67	43	57	65	32
Schools and Academies Represented					
By Investigators	2	1	1		2
By Students			3	1	1
Foreign Institutions Represented					
By Investigators	6	8	7	15	11
By Students	2	3	2	6	13

5. Cooperating and Subscribing Institutions, 1954

Cooperating Institutions

Amherst College
Brooklyn College
Brown University
Bryn Mawr College
California Institute of Technology
Children's Hospital of Philadelphia
City College of New York
Colby College
College of Mt. St. Joseph-on-the-Ohio
Columbia University
Columbia University College of Physicians and Surgeons
Cornell University
Cornell University Medical College
Drew University
Duke University
Elmira College
Emory University
Fordham University
George Washington University
Hahnemann Medical School
Harvard University
Harvard Medical School
Institute for Cancer Research
Institute for Muscle Research
Iowa State University
Johns Hopkins University
Johns Hopkins University School of Hygiene
Eli Lilly and Company
Marquette University
Massachusetts General Hospital
Morgan State College
Mount Holyoke College
National Institutes of Health
National Science Foundation
Naval Medical Research Institute
New York University, Heights
New York University College of Medicine
New York University Washington Square College
North Carolina State College of Agriculture and Engineering
Northwestern University
Oberlin College

Princeton University
Public Health Research Institute of the City of New York
Rockefeller Foundation
Rockefeller Institute for Medical Research
Rutgers University
Saint Louis University
Sloan Kettering Institute
Southwestern Medical College
State University of New York College of Medicine at Syracuse
Temple University
Tufts College
University of Chicago
University of Connecticut
University of Delaware
University of Florida
University of Illinois
University of Maryland School of Medicine
University of Massachusetts
University of Michigan
University of Minnesota
University of New Hampshire
University of North Carolina
University of Pennsylvania
University of Pennsylvania Medical School
University of Rochester
University of Virginia School of Medicine
University of Wisconsin
Vassar College
Washington University School of Medicine
Wellesley College
Wesleyan University
Western Reserve University School of Medicine
Yale University

Subscribing Institutions

American Meteorological Society
Barnard College
Belgian American Education Foundation
Catholic University of Washington
Egyptian Government Educational Bureau
Embassy of Iraq
Florida State University
Hoffman-La Roche Inc.
Institute of National Education
Long Island University
Massachusetts Institute of Technology
Michael Reese Hospital
Pennsylvania College for Women
Saint Ignatius College
Syracuse University
University of Colorado
University of Mississippi
University of Missouri
University of Nebraska
University of Pittsburgh
Vanderbilt University
Wabash College
Walter Reed Army Medical Center

6. Evening Lectures, 1954

June 25
G. B. Baerends ..... "The interaction of external and internal stimuli in releasing behaviour."

July 2
S. Meryl Rose ..... "Specific inhibition as a factor in cellular differentiation."

July 9
Hewson Swift ..... "Cytochemical studies on nucleic acids."

July 16
David R. Goddard ..... "Electron transport in plant respiration."

July 23
Britton Chance ..... "Dynamics of respiratory enzymes in oxidative phosphorylation."

July 30
Boris Ephrussi ..... "The components of the cytochrome-forming system of yeast."

August 6
Franz Moewus ..... "Function and biosynthesis of sex hormones in *Chlamydomonas eugametos*."

August 13

H. Koch ..... "The mechanism of the active transport of mineral ions by the gill epithelium of the crab *Eriocheir sinensis M.*"

August 20

G. Evelyn Hutchinson ..... "The invasion of fresh water from the seas."

7. Tuesday Evening Seminars, 1954

July 6

Allen Goldman ..... "Photochemical spectral analysis of the embryonic development of Drosophila."

Roberts Rugh and Helen Clugston .... "Radiosensitivity of the various phases of the estrus cycle in the mouse."

Max Lauffer, C. C. Brinton, Jr. and Anne Buzzell ..... "The relationship between the surface structure and the electrophoretic mobility of *E. coli* bacteria."

July 13

W. S. Vincent ..... "Protein content of starfish nucleoli."

Celia Marshak ..... "Isolation of nuclei from Asterias eggs in the germinal vesicle stage and their DNA content."

Alfred Marshak ..... "A theory of the biological role of DNA."

July 20

E. Pteemann Nielson ..... "Influence of pH on the rate of respiration in Chlorella."

Albert Tyler ..... "Univalent antibodies."

W. Troll and Sol Sherry ..... "The activation of plasminogen by streptokinase."

July 27

Joseph Pick ..... "Sympathectomy in frogs."

O. F. Hutter and Krista Kostial ..... "The relation of sodium ions to the release of acetylcholine,"

Bruno Kisch ..... "Electron microscopy of the sarcosomes of the heart."

August 3

P. W. Whiting ..... "Identifying gene elements in Mormoniella."

Richard C. Starr ..... "Some aspects of sexuality and genetics in desmids."

Franz Moewus ..... "On inherited and adapted rutin-resistance in Chlamydomonas."

August 10

D. R. Shanklin ..... "Ionic interaction at Fundulus ectoderm."

David Chalfin ..... "Some properties of mature and young erythrocytes."

M. H. Jacobs ..... "Some new permeability constants for the erythrocyte and their possible significance."

August 17

Carlos Eyzaguirre ..... "Activation of stretch receptors in Crustacea."

W. R. Lowenstein and O. F. Hutter .. "Sympathetic influence on neuromuscular transmission."

K. S. Cole and J. W. Moore ..... "In vivo measurements of the squid giant axon resting and action potentials."

8. Members of the Corporation, 1954

1. Life Members of the Corporation

Beckwith, Dr. Cora J., Vassar College, Poughkeepsie, New York
Billings, Mr. R. C., 66 Franklin Street, Boston, Massachusetts
Brodie, Mr. Donald M., 522 Fifth Avenue, New York 18, New York
Calvert, Dr. Philip P., University of Pennsylvania, Philadelphia, Pennsylvania
Carver, Prof. Gail L., Mercer University, Macon, Georgia
Cowdry, Dr. E. V., Washington University, St. Louis, Missouri
Dederer, Dr. Pauline H., Connecticut College, New London, Connecticut
Dungay, Dr. Neil S., Carleton College, Northfield, Minnesota
Goldfarb, Dr. A. J., College of the City of New York, New York City
Jackson, Mr. Charles C., 24 Congress Street, Boston, Massachusetts
Jackson, Miss M. C., 88 Marlboro Street, Boston, Massachusetts
King, Mr. Charles A.
King, Dr. Helen D., Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania
Lewis, Prof. W. H., Johns Hopkins University, Baltimore, Maryland
Lowther, Dr. Florence deL., Barnard College, New York City, New York
MacNaught, Mr. Frank M., Woods Hole, Massachusetts
Malone, Prof. E. F., 6610 North 11th Street, Philadelphia 26, Pennsylvania
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Warbasse, Dr. James P.
Webster, Mrs. Edwin S.
Whitely, Miss Mabel W.
Wickersham, Mr. and Mrs. James H.
Williston, Miss Emily
Williston, Prof. Samuel
Wilson, Mrs. Edmund B.
Wolfinsohn, Mrs. Sarah A.

V. REPORT OF THE LIBRARIAN

1954

In 1954, the number of currently-received journals totalled 1523 (50 new). Of these titles, there were 460 (9 new) Marine Biological Laboratory subscriptions; 607 (16 new) exchanges and 169 (10 new) gifts; 77 (4 new) were Woods Hole Oceanographic Institution subscriptions; 171 (5 new) were exchanges and 39 (6 new) were gifts.

The Marine Biological Laboratory purchased 42 books, received 63 complimentary copies (5 from authors and 58 from publishers) and 53 miscellaneous presentations. The Woods Hole Oceanographic Institution purchased 10 titles and received 5 gifts. The total number of books accessioned was 173.

The Marine Biological Laboratory completed by purchase 4 journal sets and partially completed 12 sets. The Woods Hole Oceanographic Institution completed 3 sets and partially completed 2 sets. Volumes and numbers received by gift and by exchange completed 5 sets and partially completed 9 sets.

The reprint collection gained 3614 papers of which 2168 were of current issue.

Forty-nine items were borrowed on inter-library loan, and 165 were sent to out-of-town libraries. The sale of duplicate material contributed $259.40 to the Laboratory's General Fund.

During the year, several very fine gifts were presented to the Library. Dr. R. P. Bigelow, continuing his keen interest in the Library, presented 8 books among which were several valuable scientific classics. To Dr. Bruno Kisch, grateful

acknowledgment is made of the very fine etching of Prof. L. Agassiz made shortly after his death by the artist Anna Merritt.

Other gifts included 13 books contributed by Dr. Henry Stommel, 6 by Dr. Dorothy Wrinch, and 6 by Dr. Richard Bliss. A total of 266 reprints were received from Drs. A. C. Redfield, A. Lazarow and P. W. Whiting.

The California Institute of Technology Library, at the suggestion of Dr. A. H. Sturtevant, returned to the Laboratory a valuable run of the "Biological Bulletin." This gift provided the opportunity to replenish stock which is exceedingly low.

In September, an Assistant Librarian was appointed, thus filling a position which has been vacant since 1947. The Library Committee and the Librarian have for a long time been fully aware of the need of an assistant, and it is a satisfaction to have this position re-established.

At the end of the year, the Library contained 64,272 bound volumes and 192,198 reprints.

Respectfully submitted,

DEBORAH L. HARLOW
Librarian

VI. REPORT OF THE TREASURER

A summary of the securities held in the Endowment Fund appears in the fifth exhibit. On January 1, 1955, these securities had a market value of $1,359,041 compared with $1,153,996 on January 1, 1954. The average yield on the securities was 4.87% on the book value and 3.5% on the market value.

Exhibit 1 is the report of our new auditors, Lybrand, Ross Bros. & Montgomery of Boston on the examination of our financial statements.

The second exhibit gives the Balance Sheet. It reflects the situation as of December 31, 1954, of Bar Neck Property. At that time negotiations were still being conducted for a more equitable settlement than the original amount of $75,000 fixed by the U. S. Navy. Since the first of the year an agreement was reached in the amount of $115,000 and payment has been received of the balance. From this total must be deducted expenses (legal, appraisals, etc.) amounting to $10,328.44. The Laboratory thus has received $104,671.56 net for the property.

The third exhibit is a condensed statement of operating expenditures and income. It should be pointed out that as of December 31, 1954, the Laboratory had spent $21,263.54 towards the repairs made necessary by Hurricane Carol. This expenditure is included in the statement. Since the close of the year a grant in the amount of $50,000 was received from the National Science Foundation in response to a Laboratory application for $61,500 as the total extent of our losses. Inasmuch as the rehabilitation program extended into the year 1955, additional expenditures will be reflected in the report of next year.

Again I would like to remind all who are concerned with the Laboratory of the need for their continued efforts to develop new sources of income. To an ever increasing extent the success of the Laboratory program is directly proportional to our fund raising abilities. A great deal of effort has recently gone into a brochure that is now available to describe the varied activities of the Laboratory.

Your officers and the members of the Executive Committee feel that this will be very useful to all of us in seeking contributions from individuals, foundations, and corporations which should be interested in supporting the basic research and teaching being done at the Marine Biological Laboratory.

Exhibit 1

To the Trustees of
Marine Biological Laboratory
Woods Hole, Massachusetts.

We have examined the balance sheet of Marine Biological Laboratory as at December 31, 1954 and the related statements of operating expenditures and income and current fund for the year then ended. Our examination was made in accordance with generally accepted auditing standards, and accordingly included such tests of the accounting records and such other auditing procedures as we considered necessary in the circumstances.

In our opinion, the accompanying financial statements present fairly the assets, liabilities and funds of Marine Biological Laboratory at December 31, 1954 and the expenditures and income for the year then ended.

LYBRAND, ROSS BROS. & MONTGOMERY

Boston, Massachusetts
July 15, 1955

Exhibit 2

MARINE BIOLOGICAL LABORATORY

BALANCE SHEET

December 31, 1954

Investments		
Investments held by Trustee:		
Securities, at cost (approximate market quotation $1,359,041)		$ 979,104
Cash		1,428
		980,532
Investments of other endowment funds:		
Securities, at cost (approximate market quotation $25,255)	$19,436	
Cash	8,227	27,663
Investments of unrestricted funds		198,710
		1,206,905
Plant Assets		
Land, buildings, library and equipment		2,392,533
Less allowance for depreciation (note A)		921,483
		1,471,050
Current Assets		
Cash		21,128
Accounts receivable ($13,441 from U. S. Government)		30,570
Inventories of specimens and bulletins		58,341
Prepaid insurance and other		8,359
		118,398
		$2,796,353

Note A—The Laboratory has since January 1, 1916 provided for reduction of book amounts of plant assets and funds invested in plant at annual rates ranging from 1% to 5% of the original cost of the assets.

Exhibit 2,
continued

Marine Biological Laboratory

Balance Sheet

December 31, 1954

Endowment Funds

Endowment funds given in trust for the benefit of the Marine Biological Laboratory		$ 980,532
Endowment funds for awards and scholarships		
Principal	$ 18,156	
Unexpended income	9,507	27,663
Unrestricted funds functioning as endowment		198,710
		1,206,905

Plant Liability and Funds

Mortgage payable on demand, 5%		5,000
Funds expended for plant	2,387,533	
Less allowance for depreciation charged thereto	921,483	1,466,050
		1,471,050

Current Liabilities and Fund

Accounts payable	31,275
Unexpended balances of gifts for designated purposes	2,222
Unexpended research grant	315
Current fund	84,586
	118,398
	$2,796,353

Exhibit 3

Marine Biological Laboratory

Statement of Operating Expenditures and Income

Year Ended December 31, 1954

Operating Expenditures

Direct expenditures of departments:		
Research and accessory services		$150,999
Instruction		25,161
Library, including book purchases		25,373
Biological Bulletin		15,035
		216,568
Administration and general		42,294
Plant operation and maintenance		59,793
Hurricane emergency repairs		21,264
Dormitories and dining services		114,756
Equipment purchased from current funds		7,433
		462,108
Less depreciation included in plant operation and auxiliary activities above but charged to plant funds		36,171
		425,937

Income

Direct income of departments:		
Research fees		41,927
Accessory services:		
Sales of biological specimens	$66,619	
Other credits for accessory services	27,403	94,022
Instruction fees		16,345
Library fees and income		6,869
Biological Bulletin, subscriptions and sales		16,017
		175,180
Allowance for indirect costs on research contracts		13,765
Dormitories and dining services income		92,569
		281,514
Investment income:		
General endowment	47,725	
Current fund investments	22,851	70,576
Gifts for current use		50,586
Sundry income		3,062
Total current income		405,738
Excess of operating expenditures over income		$ 20,199

Direct costs of $36,163 on research contracts and reimbursement therefor are not included in operating expenditures or income.

Exhibit 4

Marine Biological Laboratory

Statement of Current Fund

Year Ended December 31, 1954

Balance January 1, 1954		$249,043
Less:		
Adjustment of credit to account in 1953 for taking of Bar Neck Property	$ 19,536	
Amount transferred to unrestricted funds functioning as endowment as of December 31, 1954 pursuant to vote of Executive Committee of the Board of Trustees	124,722	144,258
		104,785
Excess of operating expenditures over income, 1954		20,199
Balance December 31, 1954		$ 84,586

Exhibit 5

Marine Biological Laboratory

Summary of Investments

December 31, 1954

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1954
Securities held by Trustee:					
General endowment fund:					
U. S. Government bonds	$185,244	22.5	$ 184,736	16.5	$ 5,200
Other bonds	285,747	34.8	293,654	26.2	7,902
	470,991	57.3	478,390	42.7	13,102
Preferred stocks	85,788	10.5	80,856	7.2	3,614
Common stocks	264,150	32.2	562,996	50.1	22,800
	$820,929	100.0	$1,122,242	100.0	$39,516
General Educ. board endow. fund:					
U. S. Government bonds	48,161	30.5	48,235	20.4	1,125
Other bonds	30,690	19.4	31,050	13.1	998
	78,851	49.9	79,285	33.5	2,123
Preferred stocks	27,281	17.2	26,485	11.2	1,130
Common stocks	52,043	32.9	131,029	55.2	4,956
	158,175	100.0	236,799	100.0	8,209
Total securities held by trustee	$979,104		$1,359,041		$47,725

Exhibit 5,
continued

Marine Biological Laboratory

Summary of Investments

December 31, 1954

	Cost	% of Total	Approximate Market Quotations	% of Total	Investment Income 1954
Securities of other endow. funds:					
Rev. A. Boyer scholar. fund:					
U. S. Government bonds	5,504	100.0	5,385	100.0	148
The Bio Club scholar. fund:					
U. S. Government bonds	2,062	50.6	1,955	46.7	51
Other bonds	2,012	49.4	2,234	53.3	82
	4,074	100.0	4,189	100.0	133
Allen R. Memhard Fund:					
U. S. Government bonds	1,000	100.0	978	100.0	28
Lucretia Crocker Fund:					
U. S. Government bonds	4,515	51.0	4,425	30.0	114
Other bonds	4,343	49.0	10,278	70.0	396
	8,858	100.0	14,703	100.0	510
Total securities of other endowment funds	$ 19,436		$ 25,255		$ 819
Investments of unrestricted funds:					
U. S. Government bonds	27,300	18.4	26,818	17.1	961
Other bonds	68,249	46.1	70,826	45.0	1,228
	95,549	64.5	97,644	62.1	2,189
Common stocks	52,481	35.5	59,513	37.9	2,882
	148,030	100.0	$ 157,157	100.0	5,071
Investment in Gen. Biol. Supply House, Inc.	12,700				17,780
	160,730				22,851
Real estate and mortgage	37,980				
Total investments of unrestricted funds	$198,710				$22,851

PHYSICAL AND BIOLOGICAL PROCESSES DETERMINING THE DISTRIBUTION OF ZOOPLANKTON IN A TIDAL ESTUARY[1]

JOHN P. BARLOW[2]

Woods Hole Oceanographic Institution, Woods Hole, Mass.

Plankton are at the mercy of the water movements that carry them about. These water movements also determine their physical and biological environments. As a result the study of the geographical distribution of plankton has an unique simplicity. The maintenance of an endemic plankton population and its distribution from its endemic center depends primarily on the dynamic balance between two processes: the translocation and dispersion of the population by the water movements, and the reaction of the population to the changes in environment caused by the water movements (Redfield, 1939, 1941; Redfield and Beale, 1940).

In spite of this simplicity, it is difficult in most environments to separate the effects of dispersal by the water movements on their distribution from the effects of reproduction and mortality of the organisms. The physical and analytical difficulties met in understanding and measuring the effects of these processes are, however, lessened by the characteristics of many tidal estuaries.

Estuaries are environments for which the land provides well defined boundaries. These boundaries permit a useful simplification in that the three dimensions in which events take place in the open sea can often be reduced to the two dimensions of a vertical plane in the land-sea axis of the estuary. Moreover, the rivers that drain into estuaries supply fresh water in a continuous and measurable fashion. This fresh water can become a useful tracer by which the rate of circulation of both fresh and salt water can be measured. The recent studies of the circulation in estuaries by Tully (1949), Ketchum (1951a, 1951b), Ketchum, Redfield and Ayers (1951), and Ketchum and Keen (1953) have given realistic descriptions of the circulation based on the behavior of this fresh water.

These studies show that there must be a net seaward movement of mixed salt and fresh water through an estuary adequate to remove the fresh water added by the river. This seaward movement must tend to transport the weakly swimming planktonic organisms. Ketchum (1954) has shown that the rate of this circulation determines the rate at which a plankton population must reproduce in order to maintain itself in an estuary. He has pointed out that the vigor of the circulation may thus limit the kind of plankton that is able to maintain an endemic population in an estuary. Bousfield (in press) has shown that in the Miramichee estuary the distribution of barnacles is influenced by seaward movements of mixed fresh and salt water and the landward counter current associated with it. Huntsman (1954) has used the estuarine circulation in the Bay of Fundy to explain the distribution of the herring populations there.

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This net seaward transport through an estuary implies that a population living in any part may be increased by additions from landward as well as decreased by losses to seaward. A knowledge of this circulation permits estimates to be made of the net rate at which a population in any part of the estuary is augmented or reduced by the physical processes. If the population is to be maintained, it must be increased or decreased by growth or mortality to offset the effects of the physical processes. Hence, if the rate of the circulation is taken into account, it is possible to determine the biological processes that underlie the distribution of plankton.

The distribution of zooplankton in a small estuary, Great Pond, near Woods Hole, Mass., has been studied. Rates have been computed at which some typical endemic zooplankton must have grown or died to maintain their distribution in face of the circulation. The rates of reproduction and mortality of these same zooplankton have also been estimated in various parts of the estuary from rates of increase or decrease of portions of the population isolated in bottles. There have thus been two independent estimates of the biological processes that maintain the distribution of these zooplankton. It is possible to give some understanding of both the physical and biological processes that determine the distribution of these zooplankton in Great Pond.

These investigations in Great Pond were suggested by Dr. Alfred C. Redfield. The author owes much to him for his continued encouragement, advice and critical analysis of this work. The author is also indebted to Dr. Bostwick H. Ketchum for his general interest in this problem, and his patient help in understanding the relations between plankton and the circulation in estuaries.

Mr. Edward M. Hulburt supplied much valuable information from his studies on the phytoplankton of the pond and helped greatly in the collection of the zooplankton samples.

Methods

Zooplankton sampling

Great Pond (Fig. 1) is a small tidal estuary in the northern shore of Vineyard Sound. It is 3,300 meters long, with a maximum width of about 300 meters and a maximum depth of a little over two meters. The topography of its basin divides the pond into the several parts, the arm, narrows, main pond, shallows and entrance, as shown in Figure 1. The stations from which plankton samples were regularly taken are also shown. A full description of the pond has been previously given (Barlow, 1952).

Regular surveys of the plankton of Great Pond were made at 10- to 15-day intervals during the spring and summer of 1950 and at longer intervals during the following winter and spring. All zooplankton samples were taken with the quantitative plankton sampler (Clarke and Bumpus, 1950). Silk nets of No. 10 mesh (nominal aperture 0.158 mm.) were used except for samples taken in February, 1951, when the water was so turbid that it was necessary to use No. 2 mesh nets (nominal aperature 0.366 mm.).

On each survey samples were usually taken from the six stations in the pond and a station in the adjacent part of Vineyard Sound. At all stations a surface

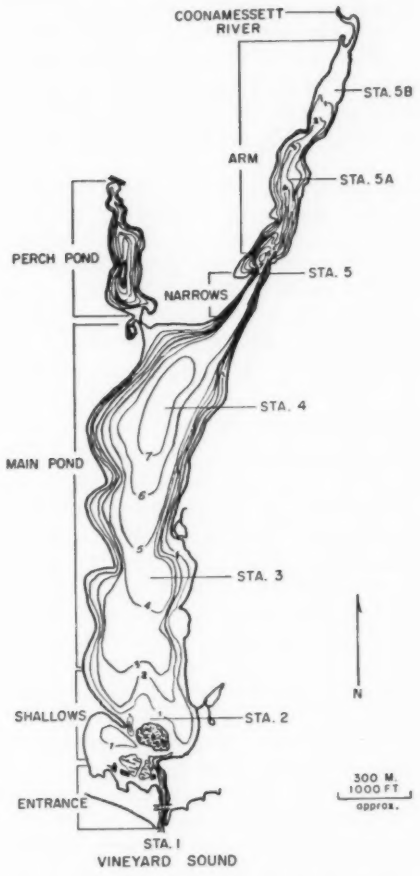

FIGURE 1. Bathymetric chart of Great Pond; depth in feet.

sample was collected by towing a sampler just below the surface. At stations 4, 5 and 5a, another sample was taken simultaneously as close as possible to the bottom. Tows were made, as far as possible, transverse to the long axis of the pond and at a velocity of two to three knots. The cyclometer readings indicated that usually two to three cubic meters of water were filtered for each sample.

Aliquots of each sample were counted under a dissecting microscope. The size of the aliquots was adjusted so that 50 to 100 of each of the important forms were counted. The concentrations in numbers per cubic meter have been computed from the numbers counted in these aliquots and the volume of water filtered for each sample.

The estimates of abundance of plankton are based on counts of single aliquots from single samples. The representativeness of such counts was tested by duplicate aliquots of a series of ten successive samples taken within two hours at station 3. Analysis for the variance between counts and the variance between hauls by the method of Silliman (1946) indicated that a sample could be considered to be significantly different from another only if it were less than half or more than twice the size of the other.

The samples have usually included so much detritus that the usual direct measurement of the weight or volume of the zooplankton could not be used. Instead, the average dimensions of each organism were determined in several representative samples. Each organism was considered to approximate in shape a simple geometric solid, and its average volume was calculated for each of these representative samples. These calculated average volumes were similar to those arrived at by more elaborate methods by Lohmann (1908) for the same or similar organisms. They have been used to compute the "calculated volume" of zooplankton in each sample.

Experiments with isolated water samples

A number of experiments were made with natural populations of phytoplankton and zooplankton imprisoned in bottles in order to study grazing rates, multiplication and growth. Water was pumped from the desired depth into a large wooden tub. After thorough mixing, one-liter aliquots of this water were poured alternately into a clean five-gallon carboy and through a No. 10 net. When the bottle contained 15 liters it was stoppered and moored where the sample had been taken. Thus filled, the bottle floated with its neck awash, and the water in it was stirred and aerated by wave motion. The sample collected in the net was preserved as a control. Usually bottles were filled and moored at five or six places in the pond. They were harvested after two or three days by pouring their contents through a plankton net. Occasionally some species not usually abundant in the pond would become very abundant in the bottle. Usually, however, the species composition changed very little during the course of an experiment.

As a measure of grazing rate this method was not entirely satisfactory, partly because large changes in the number and sizes of the animals during the course of the experiments made the results difficult to interpret. However, the multiplication and growth of the zooplankton in some of the jugs was so great that some conclusions could be drawn about the response of the animals to the differences in environment in different parts of the pond.

Results

The zooplankton population of Great Pond

There have been a number of studies of the neritic plankton fauna of the Vineyard Sound region. Among these are Fish's (1925) seasonal study of the plankton of Woods Hole harbor and Wilson's (1932) description of the copepods of the Woods Hole region including several smaller estuaries. Deevey (1948) has described a population in Tisbury Great Pond, Martha's Vineyard, that was very similar in composition, seasonal distribution and abundance to that found in the central part of Great Pond.

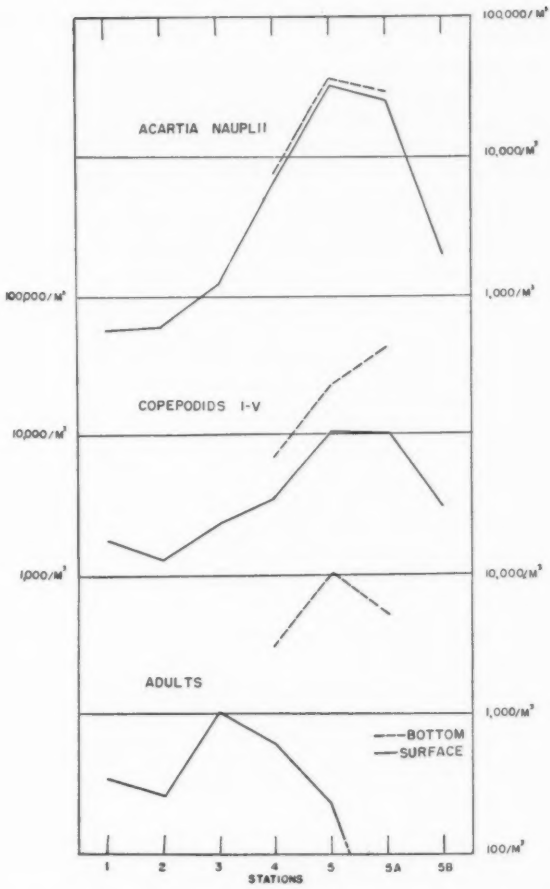

FIGURE 2. Distribution of *Acartia tonsa* copepodids and *Acartia* spp. nauplii in June 1950. Mean of surveys of June 5, 15 and 30.

The principal constituents of the catches from both the Sound and the pond have almost always been small neritic copepods, usually species of *Acartia*. During the late spring and summer these copepods were so much more abundant in parts of the pond than in the Sound that they must have been living endemically somewhere in the pond. The distribution of *Acartia tonsa* copepodid stages and *Acartia sp.* nauplii in the month of June is shown in Figure 2. The species of *Acartia* nauplii has not been determined, but since *A. tonsa* was by far the most abundant *Acartia* in June, it may be presumed that most of the *Acartia* nauplii were of *tonsa*. In addition to these copepods, several other endemic plankton organisms were abundant in this period. The cladoceran *Podon polyphemoides* and species of the

rotifer *Synchaeta* were especially numerous in some parts of the pond but were scarce or not found in the Sound.

In contrast there were only a few forms, such as the copepod *Centropages hamatus*, that were more abundant in the Sound than in the pond.

The seasonal change in abundance of plankton has been summarized in Figure 3. This figure shows the seasonal distribution of the calculated volumes of all zooplankton except medusae, ctenophores, and gastropod veligers. The medusae and ctenophores were omitted because of their high water content. The gastropod veligers were omitted because their distribution must at least in part be determined by the distribution of the adult gastropods. Other temporary plankton made but small contributions to the calculated volumes shown in Figure 3.

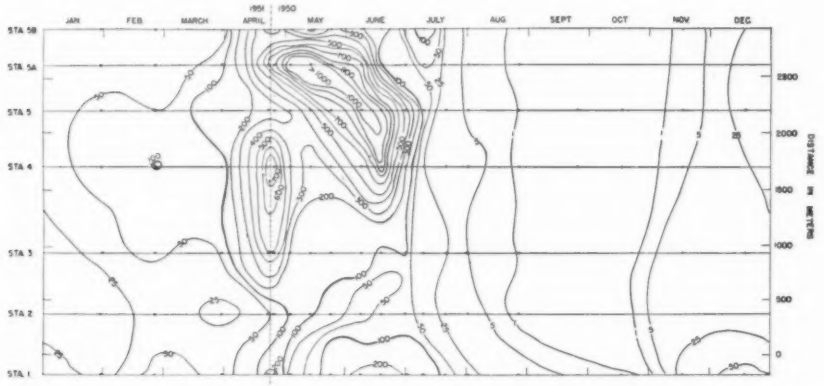

FIGURE 3. Seasonal distribution of zooplankton volume. Calculated volume in $mm.^3/M^3$. Gastropods, hydromedusae, and ctenophores not included. Mean of surface and deeper samples at Stations 4, 5 and 5a. Closed circles indicate observations. Distance in meters from entrance shown at right.

The fall and winter were periods of least abundance in both the pond and the Sound. The population increased from about the first of April through the spring to the period of greatest abundance in the early summer. In the latter part of July in both 1950 and 1951 there was a dramatic decrease in the abundance of all kinds of zooplankton in the pond. There were no obvious changes in physical, chemical or hydrographic conditions accompanying this decrease. In both years it coincided with the appearance of numbers of *Mnemiopsis leidyi*. These ctenophores were so poorly preserved that they could not be counted in the 1950 samples. Examination of some fresh samples in 1951 showed that at Station 5 they increased from less than one per cubic meter in early July to 35 per cubic meter near the end of July.

Since *Mnemiopsis* is known to be a zooplankton predator (Nelson, 1925), it seems possible that the late summer decrease in zooplankton in Great Pond was due to its depredations. Although Deevey (1948) found a similar late summer decrease in Tisbury Great Pond, she found no ctenophores in her samples. The

number of zooplankton in Great Pond did not increase substantially again during the remainder of the year.

The greatest concentration of zooplankton in the pond was found on June 5, 1950, in the deeper water at Station 5a where there were 346,000 organisms per cubic meter having a calculated volume of 2,500 mm.^{3} per M^3. The smallest concentrations of plankton in the pond and Sound were found on September 18, 1950, when there were less than 100 organisms per cubic meter, having a calculated volume less than one mm.^{3} per M^3 at Station 5a. Thus there was a several thousand-fold seasonal decrease in both number and volume of zooplankton in the pond.

The concentrations in the Sound have usually been quite different from those in the pond. On June 5 the zooplankton in the Sound was nearly at its maximum, yet it was only 1/72 in numbers and 1/38 in volume of that in the pond. The zooplankton was never as low in the Sound as in the pond. At the time of the September minimum in the pond the zooplankton was about 20 times greater, in both numbers and volume, in the Sound. The winter minimum was about 1/15 the summer maximum in the Sound.

Vertical distribution of plankton in Great Pond

There were usually no significant differences in the concentrations of *Acartia* nauplii at the surface and near the bottom. The juveniles and adult copepodids were, however, found in significantly greater concentrations near the bottom than at the surface at some stations. The difference in surface and bottom concentrations did not appear to be related to the time of day when the samples were taken, as shown by samples taken near midday and midnight of the same day. The distribution of zooplankton does not, therefore, seem to be greatly affected by vertical diurnal migrations. Large differences in surface and bottom concentrations were found when and where there were large differences in surface and bottom salinities. It is probable that the passive sinking of the plankton from the less buoyant brackish surface layer and then active avoidance of this layer are more effective in influencing the concentration of the heavier and more active forms than is a migration influenced by light.

Horizontal distribution of plankton in Great Pond

Tidal exchanges of salt and fresh water play a large part in effecting the salinity distribution in many tidal estuaries. Ketchum (1951b) has given an empirical method for estimating the changes of salt and fresh water in tidal estuaries. This method has been used to estimate the tidal exchanges that account for the average summertime salinity distribution in Great Pond (Barlow, 1952). These tidal exchanges must affect the horizontal distribution of plankton as well.

Ketchum, Ayers and Vaccaro (1952) have pointed out that if organisms such as coliform bacteria were introduced at a constant rate with the river water, their eventual distribution within an estuary would be similar to that of the fresh water, providing there were no net reproduction or mortality of the organisms. It may also be pointed out that these tidal exchanges must tend to disperse organisms landward. Hence, if plankton were present in constant numbers in Vineyard Sound its eventual distribution in Great Pond would be similar to that of the salt

water, providing there were no net reproduction or mortality of the organism as it was dispersed through the pond.

Estuaries are not uniform environments. The salinity of Great Pond, ranging as it does from about 32 $^{o}/_{oo}$ in Vineyard Sound to zero at the river mouth, provides in itself a significant environmental gradient. There must be similar gradients of other conservative environmental factors present in different quantities in the salt and fresh water.

It seems unlikely that there can be many organisms whose reproduction, growth and survival are not affected by such large environmental differences. We have found no zooplankton in Great Pond whose distribution landward or seaward from its center of abundance is consistently similar to that of the salt or fresh water. The distribution of the zooplankton is, therefore, determined not only by the exchanges of salt and fresh water but also by its own reproduction, mortality, and its specific behavior.

Ketchum, Ayers and Vaccaro (1952) have considered the effects of tidal exchanges and mortality on the distribution of coliform bacteria in the Raritan River. They have shown that after m tidal cycles the concentration C_n of coliform bacteria in a segment n of the river equal in length to one tidal excursion is

$$C_n = (C_0)_n \cdot r_n \cdot \frac{1 - (1 - r_n)^m e^{mk}}{1 - (1 - r_n)e^k}, \tag{1}$$

in which $(C_0)_n$ is the concentration of bacteria entering the segment n from the next landward segment and r_n is the exchange ratio of the segment n. The term e^k is the coefficient of net population change due to reproduction and mortality.

Since predation and the bactericidal action of sea water were always greater in their effects than reproduction, the sign of the coefficient k of the coliform bacteria was always negative. In some parts of the estuary reproduction will, however, tend to increase the size of the populations of many estuarine organisms. The sign of the coefficient k may thus often be positive.

Equation (1) can be simplified (see Ketchum, Ayers and Vaccaro, 1952) and solved for e^k to give

$$e^k = \frac{C_n - (C_0)_n(r_n)}{C_n(1 - r_n)}. \tag{2}$$

The simplification is valid only when $(1 - r_n)e^k$ is less than unity.

The term e^k in equation (2) defines the rate at which the population in a given segment must be increased to maintain it there at the concentration C_n in face of additions or withdrawals by tidal exchanges. This rate will be called the "replacement rate." If the sign of k in a specific segment of the estuary is positive, it implies that the population within the confines of that segment must be increasing by reproduction or by additions from deeper water below the limits of the segment to maintain the observed concentration in spite of the losses due to tidal exchanges. If the sign of k is negative, it implies that the population within the segment must be decreasing by mortality or by losses to the deeper water to maintain the observed concentration in spite of the gains due to tidal exchanges.

Estimates of the rates of the processes that maintain the observed distribution of Acartia

The replacement rates necessary to maintain a population at an observed steady state concentration C_n in any segment of Great Pond may be readily computed if the distribution of the population is well enough known that the contribution $(C_0)_n$ from the next landward segment can be estimated.

Both *Acartia* nauplii and *Acartia tonsa* copepodids were abundant and widespread throughout June and July, 1950. The *Acartia* copepodids were found in about the same abundance at the several stations in the pond on two successive surveys, June 30 and July 10, and the nauplii on three successive surveys, June 5, 15 and 30.

The nauplii were most abundant at the northern end of the main pond and in the arm, at stations 4, 5 and 5a, during June. An analysis of variance (Snedecor, 1946) of the June counts of nauplii at these three stations showed that none of the differences between surface and bottom concentrations, or between surveys, were significant. There were similar differences in abundance of *Acartia* copepodids in the June 30 and July 10 surveys. The distribution of these two forms appears to have been approximately in a steady state for the periods covered by these successive surveys.

The concentrations of *Acartia* nauplii and copepodids in the tidal excursion segments of the pond were estimated from the data of these surveys and are shown in Figures 4 and 5. At stations where samples were taken at both surface and bottom the average of surface and bottom concentrations has been considered to most nearly represent the average concentration in the mixed layer. The replacement rates necessary to maintain in each segment these observed steady state concentrations of *Acartia* nauplii and copepodids have been computed, using the relationship presented in equation (2). The replacement coefficients are shown in Figures 4 and 5. Each of these coefficients has been plotted against the mean salinity of its respective segment.

At salinities less than 15 $^{o}/_{oo}$, representing values from the arm landward of station 5, the replacement coefficients were usually positive and relatively large. These large coefficients indicate that the concentrations of both nauplii and copepodids in the mixed layer of the arm were being maintained in face of seaward transport by very rapid replacement.

At the intermediate salinities of from 15 $^{o}/_{oo}$ to 25 $^{o}/_{oo}$, representing values from the narrows and the northern end of the main pond, the replacement coefficients were also usually positive but much smaller than in the arm. These coefficients indicate that, even though the populations were large in this part of the pond, they were being maintained by replacement at a moderate rate.

At salinities above 25 $^{o}/_{oo}$, representing values from the southern half of the main pond and the shallows, the replacement coefficients were nearly all negative. These coefficients indicate that the *Acartia* must have been removed from this part faster than could be accounted for by exchanges with the Sound.

Production rate in isolated water samples

The replacement rates, e^{k}, calculated from equation (2) represent the rates of change in the population by reproduction and mortality which, under steady state conditions, balance the gains or losses by tidal exchanges. These rates would

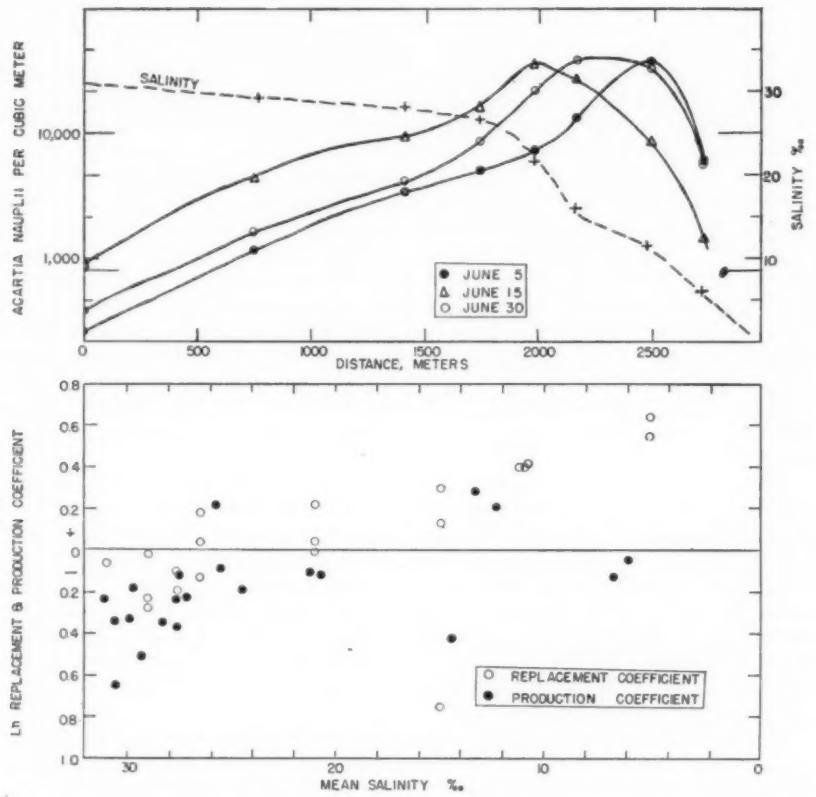

FIGURE 4. Distribution of *Acartia* nauplii (above) and replacement and production coefficients for *Acartia* nauplii (below). Replacement coefficients calculated from distributions of June 5, 15 and 30, 1950. Natural logarithms of replacement rates, e^k, plotted against mean salinity in tidal excursion segments. Production coefficients calculated from bottle experiments of June and July 1951. Natural logarithms of production rates, e^k, plotted against salinity in the bottles.

also reflect the effects of vertical exchanges between the mixed layer and the deeper layer, which may or may not be significant. The replacement rate thus gives an estimate of the rate at which the population would increase or decrease if purely horizontal tidal exchanges were eliminated.

An independent estimate of the value of e^k may be obtained by actually isolating a sample of water with its natural population from tidal exchanges and noting the rate at which its population actually changes when maintained under otherwise natural conditions. These two estimates should agree unless vertical exchanges are significant.

The change in numbers in an isolated parcel of water may be represented by

$$(C_0)e^{kt} = C_t, \tag{3}$$

in which C_0 is the initial concentration of the organism and C_t the concentration after t tidal cycles. The value of e^k determined from equation (3) will be called the "production rate," in distinction from the value obtained from equation (2) which is referred to as the replacement rate.

When applied to the numbers of some particular stage in the life history of an organism, as in the present case, these production rates are not, of course, true

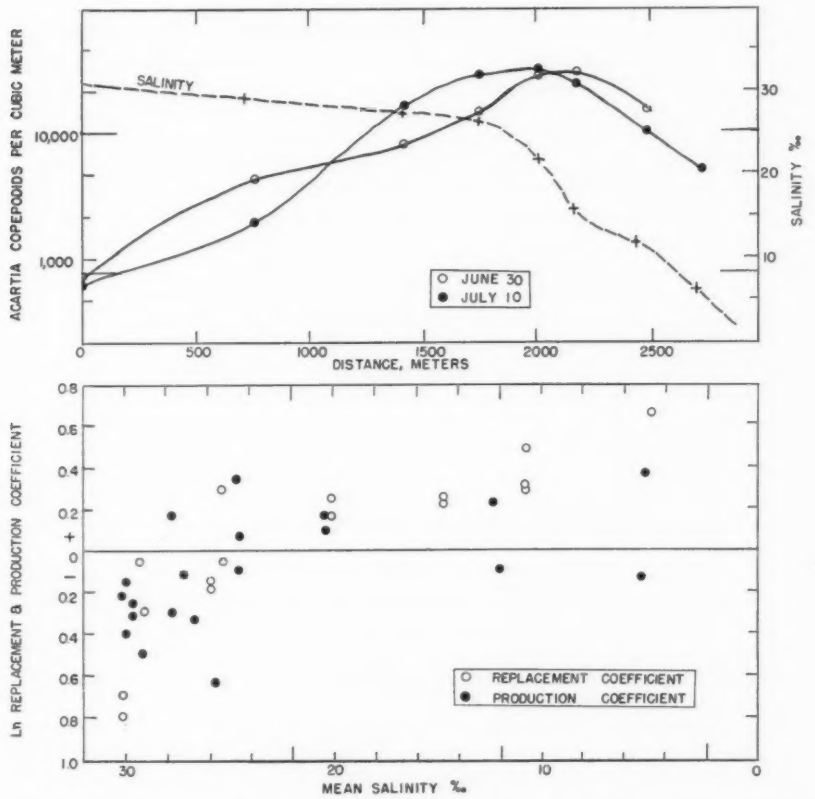

FIGURE 5. Distribution of *Acartia tonsa* copepodids (above) and replacement and production coefficients for *Acartia tonsa* copepodids (below). Replacement coefficients calculated from distributions of June 30 and July 10, 1950. Natural logarithms of replacement rates, e^k, plotted against mean salinity in tidal excursion segments. Production coefficients calculated from bottle experiments of June and July, 1951. Natural logarithms of production rates, e^k, plotted against salinity in the bottles.

measures of the rate of change of the entire population; they refer rather to the changes resulting from hatching, moulting, and the mortality of the particular stage in question.

In the bottle experiments portions of the *Acartia* population were effectively isolated from all exchanges with the surrounding water, but were maintained under natural conditions of temperature and illumination. Production coefficients have been computed from the change in numbers of *Acartia* nauplii and copepodids during these experiments using equation (3). Since it is reasonable to assume that the environmental conditions in the pond were essentially the same in 1950 and 1951, these production coefficients have also been shown in Figures 4 and 5 for comparison with the replacement coefficients. The production coefficients have been plotted against the salinity of the water enclosed in each bottle.

Comparison of the production rates with the replacement rates shows that in the main pond these two estimates of the rates of the biological processes are in general agreement. The greatest production rates were at the intermediate salinities of 10 to 15 $^{0}/_{00}$, typical of the narrows and northern end of the main pond. There the populations often reproduced fast enough to double themselves in less than two days. The agreement between the replacement and production rates indicates that there the *Acartia* population was replaced in the mixed layer by reproduction and by horizontal tidal exchanges with the population in the more landward part of the pond. The deeper layer could not have been a very potent source of recruits to the mixed layer since the *Acartia* population in the deeper layer in this part of the pond was little larger than that in the mixed layer.

At the higher salinities typical of the more seaward part of the pond the production coefficients were usually negative, showing that the *Acartia* brought in by exchanges with the landward parts of the pond were being reduced. Since the replacement rates were also negative these populations must have been reduced by some other means than by exchanges with the Sound. The hydrographic data show that most of this part of the pond was so thoroughly mixed that there could seldom be a distinct deeper layer which could receive the *Acartia* removed from the mixed layer. These populations must, therefore, have been reduced by an excess of mortality within this part of the pond over recruitment from the landward parts of the pond.

The cause of this mortality in the southern end of the pond is not known. Similar mortality rates have, however, been shown for lake zooplankton caught in outflowing rivers. Galtsoff (1924) has suggested that the mortality of this lake zooplankton may often be caused by the strong turbulence in the river. The strong turbulence in the shallows and entrance may have caused an excessive mortality of zooplankton in that part of the pond.

The replacement rates at the low salinities typical of the mixed layer of the arm were much higher than the production rates. These replacement rates indicate that the populations in this part of the pond must have been replaced very rapidly if they were to be maintained there. If they were to be replaced by reproduction alone, they would have had to double or triple themselves each day. As the rates of increase in the bottle experiments suggest, these are improbably high rates for an organism such as *Acartia*. A large part of the copepod population in the mixed layer may have been recruited by vertical mixing or migration from the deeper layer. This is possible because the deeper layer usually had a much

richer population than the surface layer. The population in the deeper layer in turn must have been replaced rapidly if it were to provide a continuous supply to the mixed layer. This replacement may have been provided by landward transport in a deeper counter current from the regions of maximum abundance near Station 5.

The comparison of the replacement and production rates made above has shown that a high rate of reproduction served to maintain the populations in the narrows and northern end of the main pond. At higher salinities, in the more seaward parts of the pond, there was a decrease in the rate of the biological processes. The small populations in these parts of the pond were a result of mortality and exchanges with the Sound. At the lowest salinities, in the surface layer of the arm, the biological processes are insufficient to maintain the populations found there. The discrepancy between the rate at which these organisms reproduce and the rate at which they must be replaced in these parts of the pond appears to be made up by recruitment from the deeper layer.

Discussion

The distribution of plankton does not in itself give a reliable definition of the limits of the region in which the organism is able to maintain itself endemically by its own reproduction and growth. The analysis of the processes that maintain the distribution of *Acartia* has shown that nowhere in the surface mixed layer of Great Pond did this copepod maintain itself wholly by local reproduction. The population in the arm could not have maintained itself without continuous additions from the deeper layer. Even the population in the mixed layer of the northern end of the main pond was dependent to some extent on additions from the more landward parts of the pond. Mortality must have far exceeded reproduction to account for the observed concentrations of *Acartia* in the seaward part of the pond.

If any holoplankton organism is to attain an endemic relation to a specific region, it is necessary that the biological processes that maintain it balance the physical processes that disperse it. This necessity is lessened but not evaded by organisms seeking regions in which dispersion is less rapid such as the deeper layer in Great Pond. In the final analysis this necessity can be met only by a sufficient rate of population increase. The fitness of a plankton organism to establish an endemic relation to a specific region depends on its rate of reproduction and growth and on its survival in the environment of that region. Smaller organisms often have the higher reproductive rates and shorter periods of immaturity that contribute to high rates of population increase. It is perhaps significant that all the endemic plankton organisms of an estuary such as Great Pond are small.

The physical processes of advection and diffusion set the minimum rate at which a plankton organism must be able to increase to maintain itself endemically. In Great Pond this minimum rate was achieved by several zooplankton in the summer during the time when temperature is high and the phytoplankton abundant. In the late summer and early fall these zooplankton appear to be unable to reproduce fast enough to maintain their numbers, perhaps because of the depredations of ctenophores.

The higher salinities in the pond in the winter suggest that there may have been some change in the physical processes that dispersed the plankton. Whether the

rate of these physical processes increased or not, the biological processes of reproduction and growth must have been decreased by the lower temperatures and sparser phytoplankton of the winter to the point where they may have been insufficient for any zooplankton to maintain themselves in the estuary by these processes. The zooplankton would, however, be able to tide themselves over such periods by the formation of resting stages on the bottom. It was not until the temperature and the phytoplankton began to increase in the spring that there was any clear evidence that any of the zooplankton in the pond was able to increase sufficiently to maintain itself endemically.

Summary

1. The zooplankton of a small estuary, Great Pond, has been described. It has been shown that in the summer there were endemic populations of several zooplankton of which the copepod *Acartia* was the most numerous and widespread.

2. The rates at which *Acartia* must have been replaced to maintain its numbers in the surface mixed layer of Great Pond in face of dispersion by the tidal exchanges have been calculated. These rates are compared with the rates at which *Acartia* increased or decreased when isolated in bottles immersed in the pond.

3. This comparison shows that in the most landward parts of the pond the population in the mixed layer could not reproduce fast enough to maintain itself and was dependent on recruitment from the deeper layer. In the central part of the pond reproduction alone was sufficient to offset the seaward transport by the tidal exchanges. In the most seaward parts of the pond, mortality was so great that the small population was maintained only by transport from the landward parts of the pond.

4. Thus the comparison reveals the contribution of reproduction, growth and mortality and of tidal transport to the maintenance of the observed distribution of *Acartia*. Seasonal changes in these physical and biological processes account for the seasonal changes in the distribution of zooplankton in Great Pond.

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THE ANNUAL REPRODUCTIVE AND NUTRITIONAL CYCLES IN TWO WESTERN SEA URCHINS[1]

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A previous study of the reproductive state of the purple sea urchin, *Strongylocentrotus purpuratus*, suggested the possibility of more than one cycle of gonadal growth during the year 1952–53 (Lasker and Giese, 1954). The reproductive state was assayed by determining the ratio of the volume in ml. of gonadal tissue to the wet weight in grams of the sea urchin, this ratio being called the gonad index (GI). In order to obtain more decisive data on gonadal growth, in the present study the gonad index was determined twice monthly for a year (1953–54) except for a few times when poor tides made collection hazardous. Furthermore, samplings were made at two localities, Pescadero Point on the Monterey Peninsula, and Yankee Point, a more exposed location on the Pacific Ocean about 10 miles farther south, since in this way the effect of different local conditions might be checked. Each sample consisted of 20 animals; thus gonad indices were obtained for 20 specimens per fortnight from each of two collecting grounds.

A monthly study of the gonad index of the large sea urchin, *Strongylocentrotus franciscanus*, was also made in 1953–54, in order to see to what extent its reproductive cycle resembled that of the smaller urchin. Collection was limited in this case not by tides but by conditions on the water; when the surface was rough, collection from a boat became too difficult. Therefore, though no collections were missed, the time intervals between collections were not perfectly regular. Ten specimens of *S. franciscanus* were analyzed each month, from catches obtained with a large net dragged by rope in about 15 feet of water near the Hopkins Marine Station.

The previous study involved the determination of nutritive state as given by the body fluid non-protein nitrogen (NPN) and reducing sugar (RS) in every specimen of *S. purpuratus* whose gonad index was determined. The same thing was done in this study for every specimen of *S. franciscanus*. Time limitations prevented the continuation of NPN and RS measurements in *S. purpuratus*, however, only occasional samples being taken.

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Experimental Data

1. *The reproductive cycle of S. purpuratus*

The data for the gonad index of the purple sea urchin for the year 1953–54 are given in Figures 1 and 2, along with similar data for 1952–53. It is evident that only a single breeding cycle occurred at Pescadero Point during 1953–54, whereas the data from the same location in 1952–53 suggest the possibility of a second cycle. At Yankee Point there was a dip in February, 1954 which appears to correspond to that at Pescadero Point in February, 1953; but this may be spurious, since it is due to the presence of 8 spawned-out animals among the 20 of the sample, the

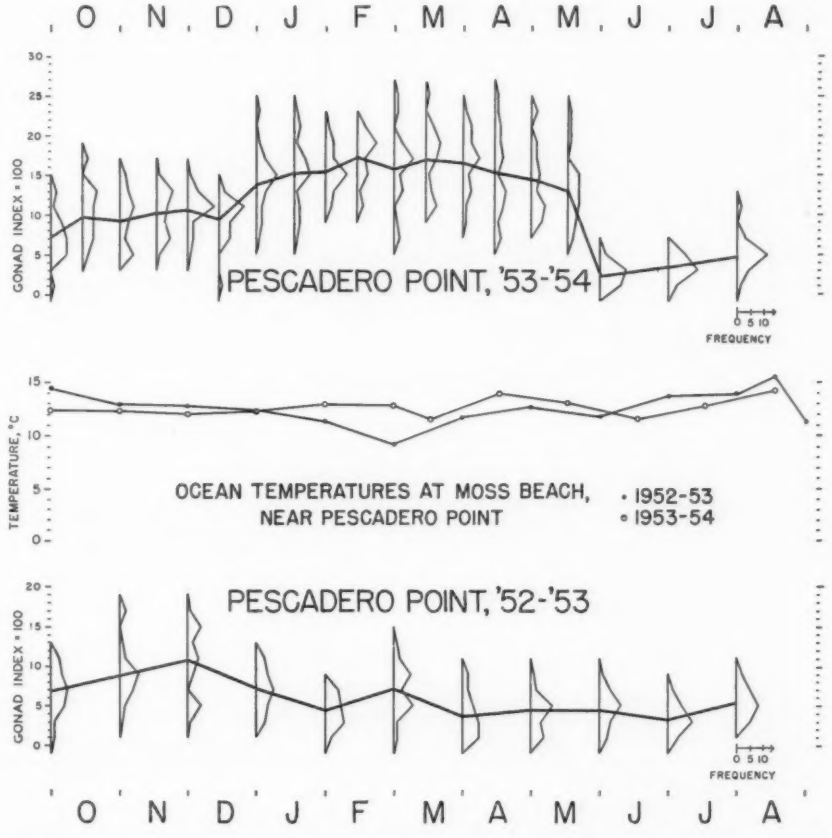
O N D J F M A M J J A
GONAD INDEX × 100
PESCADERO POINT, '53-'54
0 5 10
FREQUENCY
TEMPERATURE, °C
OCEAN TEMPERATURES AT MOSS BEACH, NEAR PESCADERO POINT
• 1952-53
• 1953-54
PESCADERO POINT, '52-'53
GONAD INDEX × 100
0 5 10
FREQUENCY
O N D J F M A M J J A

Figure 1. Reproductive cycle of *Strongylocentrotus purpuratus* in succeeding years, and corresponding ocean temperatures. The great difference in ripening is correlated with an opposite temperature gradient in the winter months. Each sample distribution is plotted in full, and a solid line joins the sample means. The frequency scale is the same for each distribution.

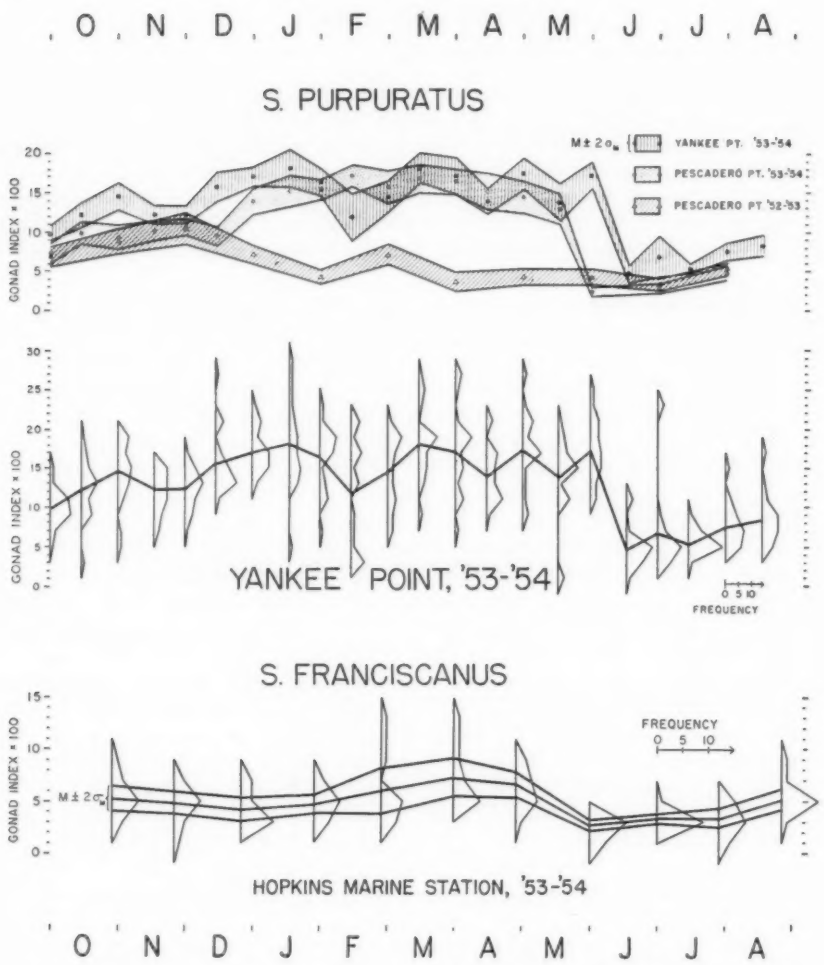

FIGURE 2. Reproductive cycles of *S. purpuratus* and of *S. franciscanus*. The bands in the top chart represent approximately 95 per cent confidence limits about the sample means; thus high significance of the differences between 1952–53 and 1953–54 means is clearly indicated. A similar band is drawn in the bottom chart to show the degree of significance of the seasonal variations in *S. franciscanus*.

other 12 being fully as gravid as the animals at the peak periods. All things considered, it therefore seems more reasonable to assume a single annual breeding cycle for the species with random variations, than a consistent double or multiple cycle.

The most striking difference between the 1952–53 and the 1953–54 cycles is the much higher gonad indices for the latter year. The gonads reached almost

double their 1952–53 size. It would be very interesting to know to what factor this greater growth might be attributed, for example whether it was a better food supply or a more favorable temperature. Examination of the temperature data shown in Figure 1 indicates that the ocean temperatures, in general, were higher

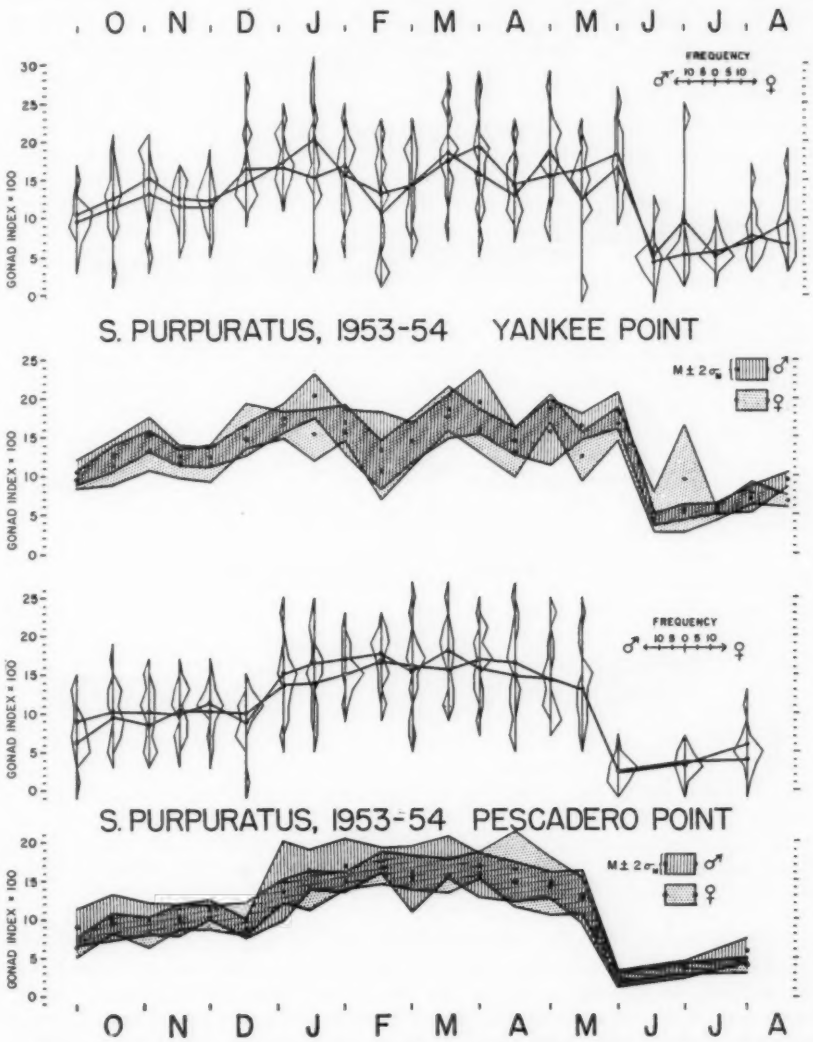
O N D J F M A M J J A
FREQUENCY
10 8 6 4 2 0 2 4 6 8 10
GONAD INDEX × 100
S. PURPURATUS, 1953-54 YANKEE POINT
M ± 2σM
S. PURPURATUS, 1953-54 PESCADERO POINT
O N D J F M A M J J A

FIGURE 3. Male and female reproductive cycles of *S. purpuratus* (1953–1954). The overlap of the 95 per cent confidence bands throughout the year indicates a close correlation of male and female cycles.

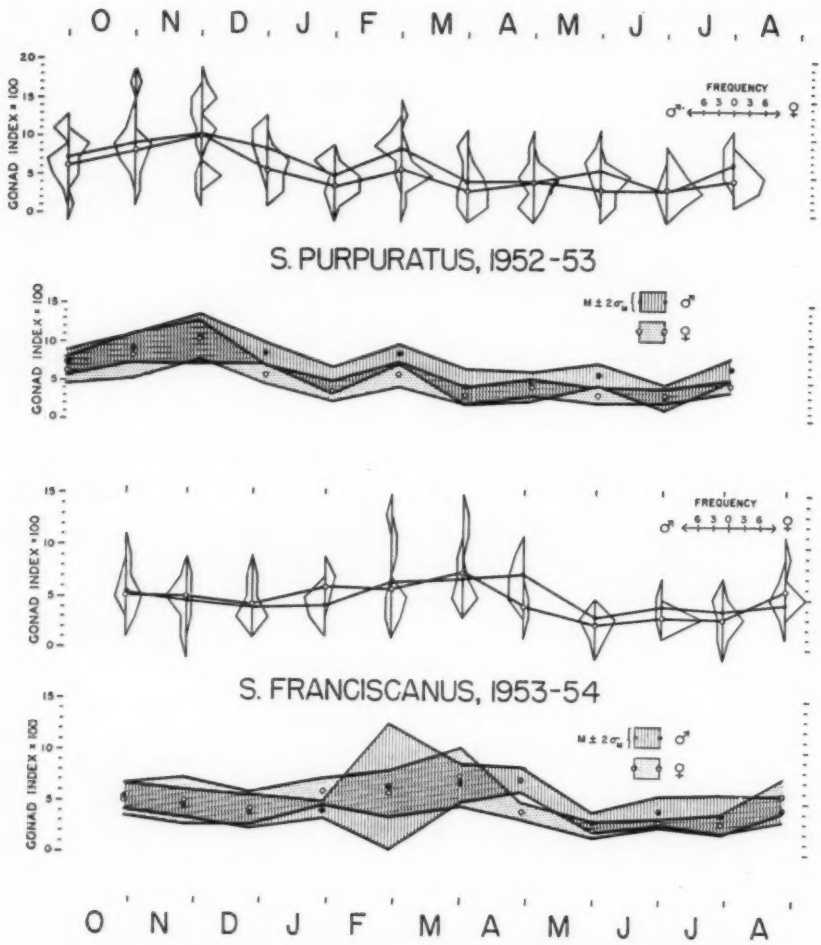

FIGURE 4. Male and female reproductive cycles of *S. purpuratus* (1952–1953), and of *S. franciscanus* (1953–1954). Again no significant difference between male and female cycles is shown.

in 1953–54 than in 1952–53. This could have had an indirect effect on the urchins by making possible a larger growth of algae. Unfortunately, no data are at present available on the algal crop for the year. Other physiological variables have been considered as possible causes of the observed difference, *e.g.* rainfall (therefore indirectly, salinity), and amount of sunlight; but there seems to be less difference between the two years on the basis of these factors than on the basis of water temperature, perhaps correlated with algal growth.

The data for the reproductive cycle in male and female urchins are separately analyzed in Figures 3 and 4. While male gametes are available for a longer period of time than female, it is evident that only a single, closely correlated cycle of gonad index occurs in both sexes.

The fertilizability of eggs was determined at each sampling, except during the peak months of 1953–54 when nearly every animal exuded great masses of gametes, which condition was previously and subsequently found to be an almost certain indication of ripeness. The criterion of ripeness in females was development of fertilization membranes in over 50 per cent of eggs in the microscope field after introduction of sperm. In males, the criterion was simply presence of sperm, there being found no instance of motile sperm which would not fertilize ripe eggs.

The difference in gonad index between the 1952–53 and 1953–54 seasons was strongly correlated with a difference in ripeness of females; in no collection during 1952–53 were a majority of the females ripe, whereas practically all females were ripe from December through May of 1953–54. The males did not provide as good a contrast, though there were many more unripe males found during the earlier year.

It is a point of interest that starvation does not appear to decrease fertility in these urchins. In fact, one of the most fertile females observed was a "spawned-out" animal which had been starved for three weeks and which appeared near death, most of its spines having fallen off. It exuded many eggs, virtually all of which developed excellent membranes within a few seconds after sperm were provided.

2. The reproductive cycle of S. franciscanus

A study of the reproductive cycle was also made for the large sea urchin, *S. franciscanus,* and the data for the gonad index are shown in Figure 2. A single cycle for the year is manifested, but the index reached its maximum somewhat later than in the purple sea urchin. The index was high for a much briefer period of time, suggesting a shorter breeding season. The gonad index of the larger species also never reached the large values found in the smaller species during the year. Perhaps because of the size of the animals, an equivalent enlargement of gonad would be beyond the aeration capacity of the body fluid, although no decrease of gonad index with increasing size was observed in *S. purpuratus.* Fertilizability of eggs of *S. franciscanus* was determined in the same way as for the smaller species, except that the eggs were followed to cleavage because the fertilization membranes were indistinct in some cases. Ripeness was found to be correlated with gonad index, better in the case of females than males, just as in *S. purpuratus.* But no such tremendous fecundity was exhibited by *S. franciscanus* as by the smaller species during 1953–54. A close correlation of male and female cycles of gonad index was found in *S. franciscanus,* as in *S. purpuratus* (Fig. 4).

3. The NPN and RS cycles in S. purpuratus

The techniques employed in measuring NPN and RS in *S. purpuratus* have been described in the previous study (Lasker and Giese, 1954). The results were reported by graphing the means of the monthly samples, but the descriptive statistical work undertaken in the present study has revealed a feature of the earlier results which calls for their analysis and further discussion. The data are represented in Figure 5, with the distribution of each of the monthly samples super-

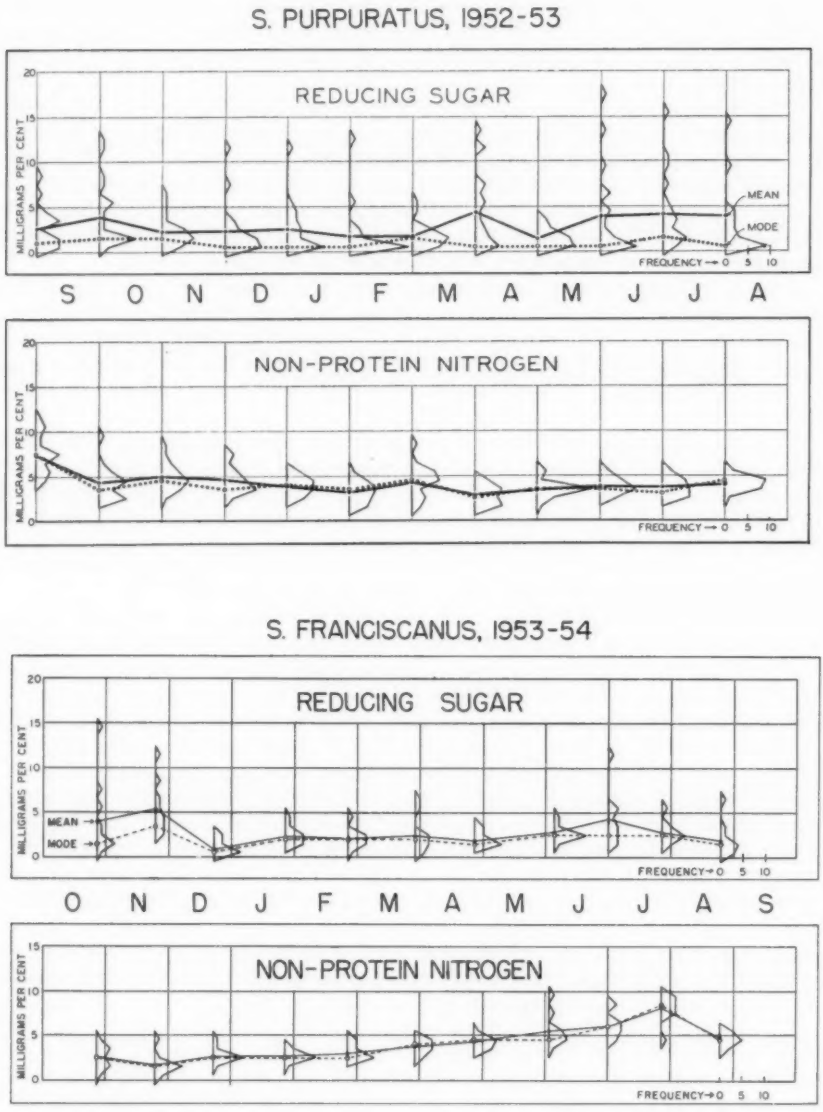

FIGURE 5. Reducing sugar and non-protein nitrogen cycles in *S. purpuratus* and *S. franciscanus*. RS distributions are positively skewed and NPN distributions are non-skewed. This is well indicated by the fact that mean is above mode in every RS distribution, but as often below as above in the NPN distributions.

posed on the graphs of means, and with the graphs of modes also included. It is clear that no highly significant seasonal variation is indicated in either NPN or RS in this species. The outstanding feature is the difference in skewness of the two kinds of distributions, the NPN being fairly normal, while the RS are highly skew.

This difference in shape of NPN and RS distributions is a fact which might have remained undiscovered had the routine statistical work of plotting the distributions not been done. It thus illustrates in a small way the importance of adequate descriptive statistical treatment of experimental data.

It is quite apparent that in the RS distributions the mean does not represent the point where the bulk of each distribution is concentrated, as it is usually assumed to do, and that the bulk of the distribution is more stable than the mean, so that there is less seasonal variability in RS than is indicated by a graph of the means. This is an instance in which the mode, which by definition follows the bulk of the distribution, provides a less misleading representation of the data than does the mean.

In the NPN distributions, by contrast, mean and mode closely coincide in almost every case, and for statistical reasons the mean is to be preferred for the representation of the data.

4. The NPN and RS cycles in S. franciscanus

NPN and RS in *S. franciscanus* were determined during 1953–54 by the same basic techniques as those employed with *S. purpuratus*, and the results are represented in the same way as for the latter species in Figure 5. The differences in skewness of distribution of NPN and RS appear in this species also. The difference is not so striking, but this may be an entirely statistical effect, owing to the smaller sample size.

Whereas no seasonal variations were clearly demonstrated in *S. purpuratus*, there was a decidedly significant increase in NPN throughout the spring in *S. franciscanus*. No correlation was observed between the growth of gonads, as measured by the gonad index, and the amount of NPN in the body fluid. The cause of the seasonal variation of NPN in *S. franciscanus* thus remains undetermined, though it may be associated with the great outgrowth of algae which occurs during the summer.

5. Total nitrogen in the filtered body fluid of the two species

Total nitrogen was measured, as well as NPN, in the filtered body fluids of a few specimens of each species, the differences being attributable to proteins. Results are shown in Table I. Technical limitations were such that accurate figures for the protein N could not be obtained; it can be said only that the protein N is a small percentage of the total, almost certainly less than 20 per cent. Thus the protein concentration, excluding clotting proteins which were always removed in filtration, is of the order of 0.5 mg. per cent.

Discussion

An annual breeding season, as judged by spawning, is believed to be common in many west coast invertebrates (MacGinitie and MacGinitie, 1949). Field observations of spawning have also established that the breeding span for a given

TABLE I

Mg % total nitrogen and protein nitrogen in the body fluid of Strongylocentrotus purpuratus and S. franciscanus

A. *S. purpuratus.* M ± $2\sigma_M$ from duplicate determinations

Total N	5.90 ± .14
NPN	5.43 ± .14
PN	0.47 ± .20

B. *S. franciscanus.* M ± $2\sigma_M$ from duplicate determinations

Total N	2.46 ± .17	4.79 ± .17	3.08 ± .17
NPN	2.04 ± .28	4.30 ± .14	2.46 ± .06
PN	0.42 ± .32	0.49 ± .22	0.62 ± .18

C. *S. franciscanus.* M ± $2\sigma_M$ from triplicate determinations

Total N	6.71 ± .31	5.76 ± .33	3.86 ± .14	5.60 ± .21
NPN	6.31 ± .09	4.90 ± .14	3.50 ± .07	5.21 ± .06
PN	0.40 ± .32	0.86 ± .36	0.36 ± .16	0.39 ± .22

species is different at different geographical locations along the coast, and at a given location, for different habitats, *e.g.,* in deeper water as compared to shallow. However, the span of the season as defined by gonad size has not been characterized in many species.[2]

In the present account which reports a series of measurements on gonads from two species of sea urchins, *Strongylocentrotus purpuratus* and *S. franciscanus,* there is indicated a single reproductive season during which the gonads gradually increase in size until, in each individual of the former species taken from a given habitat, they are so swollen with gametes as virtually to fill the body cavity. They remain in this condition until induced by some undetermined cause to spawn, and then the gonads become spent throughout the entire local population in a fairly short time. While it is likely that gonads may become replenished in the course of a season, no conclusive evidence for this was obtained, since the same animals could not be sampled successively. Spawning might give some indication of this possibility, but no satisfactory method for its induction in the laboratory was found, although chemical and electrical methods were tried (Harvey, 1939, 1954).

The factors involved in the growth of the gonads may be numerous. That temperature influences productivity is suggested by the doubled gonad volume reached during 1953–54 as compared to that of 1952–53, the former being a warmer year with no very cold spells in the water. A correlation with temperatures was reported by Stott (1931) for the sea urchin *Echinus esculentus,* spawning occurring when the sea temperature is rising in late April, May, and June. He observes the same for *Mytilus* and *Ostrea* in the same region (Port Erin). Such an effect might act indirectly by affecting the growth of the algae. More data on growth of gonads in many species of invertebrates, in addition to information on their spawning, are desirable before correlation with climatic factors can be made significant.

It is a natural presumption that cycles of nutritional components of the body fluid would be correlated with the reproductive cycle in many marine invertebrates.

[2] This has been done with vertebrates; see Bullough (1951).

This was not found to be the case with the species studied here, however. No seasonal variations in reducing sugars were found in either species; non-protein nitrogen varied seasonally in *S. franciscanus,* but no relation between the breeding and NPN cycles was established.[3]

The skewness of RS and the normality of NPN distributions in both species are facts which call for an explanation. Even neglecting the shapes, the differences in variability of these distributions suggest that NPN is loosely regulated, perhaps in association with the gonads, while RS may be entirely unregulated in the organism. As for the shapes themselves, a possible interpretation of the skewness of the RS distributions is that only a small proportion of urchins is feeding at any given time, this proportion having a relatively high RS concentration during the short period of the transfer of readily available sugars across the gut wall, while the non-feeding majority have very little RS in the body fluid, because of its rapid absorption by tissues.

Various aspects of this interpretation are subject to experimental testing. In the first place, starvation experiments should reveal the differences in degree of regulation of RS and NPN. Results of such experiments, reported in the previous paper (Lasker and Giese, 1954), demonstrate the greater regulation of NPN, and the virtually complete absence of regulation of RS.

In the second place, the role of the gonads in NPN regulation may be investigated by means of starvation experiments on gravid urchins and spawned-out urchins. Regulation should be superior in the former if the gonads play a major role. An attempt to carry out this experiment was only partially successful, because the unripe animals were not completely spawned out, as revealed at the end of the starvation period (Table II). The increase of mean NPN of specimens fed for one and two days after the starvation period shows that the regulation of NPN is rather loose.

In the third place, the proportion of specimens taken in the act of feeding on sugar-rich algae could be counted, and their RS concentrations determined separately, as a partial check on the interpretation of RS skewness; such animals should constitute a small proportion, and should have a relatively high RS concentration if the interpretation is correct. This work remains to be done but is retarded because of lack of information on the algae.

Finally, the RS skewness depends also on the rapid uptake of RS from the body fluid by the tissues. A determination of the time of withdrawal of injected glucose, reported in the previous study, indicates that over 90 per cent of the glucose is withdrawn within 4 hours; this result supports the suggested interpretation.

Other interpretations are possible, which may be in accord with the known facts in whole or in part. One is that the RS skewness may be due to the presence of a metabolic disorder, analogous to diabetes, in a small proportion of the animals. A simple experiment would decide between this and the former interpretation: If a sample of animals were fed boiled potato, only a small proportion should have a marked increase of RS if the metabolic disorder interpretation is correct, while all should have it if the other is correct. Results of the previous study (see Table I, Lasker and Giese, 1954) show marked increase in RS in three out of four animals thus fed, supporting the original interpretation, though not conclusively.

Further work remains to be done before it can be said that the shapes of the

[3] Similar results are appearing in the present season 1954–55.

TABLE II

NPN and RS in the body fluid of S. purpuratus after starvation and refeeding boiled egg white

GI*	Wet weight of urchin in grams	Weight ingested in grams	Mg % RS	Mg % NPN ($M \pm 2\sigma_M$)	Gonad N % ($M \pm 2\sigma_M$)
A. Five urchins of low GI, starved for three weeks					
3.3	70.5	0	0.6	5.3 ± 0.4	$1.93 \pm .05$
4.8	77.9	0	0.7	6.2 ± 0.4	1.92
3.1	77.3	0	0.4	5.4 ± 0.4	$1.95 \pm .42$
4.3	94.9	0	0.5	6.7 ± 0.4	$2.25 \pm .02$
2.9	91.4	0	0.8	6.1 ± 0.4	$2.07 \pm .18$
3.7 (av.)			0.6 (av.)	5.9 (av.)	
B. Four urchins of high GI, starved for three weeks					
16.7	67.1	0	1.5	2.7 ± 0.2	
15.0	92.2	0	1.9	4.2 ± 2.8	
15.8	51.1	0	3.4	5.5	
10.5	93.1	0	2.0	3.7 ± 1.0	
14.5 (av.)			2.2 (av.)	4.0 (av.)	
C. Four urchins of high GI, starved three weeks, then fed one day					
13.9	83.0	2.67	3.1	6.1 ± 1.2	
10.2	56.0	1.21	3.3	7.4 ± 2.0	
12.6	70.6	2.75	3.2	6.8 ± 0.6	
13.1	69.2	3.22	—	10.6	
12.5 (av.)			3.2 (av.)	7.7 (av.)	
D. Three urchins, starved three weeks, then fed two days					
5.4	51.8	6.50	3.5	9.2	
2.4	77.8	5.06	3.4	8.2 ± 0.2	
15.6	85.1	5.40	3.2	7.5 ± 0.2	
7.8 (av.)			3.4 (av.)	8.3 (av.)	

* GI (gonad index) as used for these data is the ratio of gonad weight in grams (instead of gonad volume in cc.) to urchin wet weight in grams.

RS and NPN distributions in these urchins are fully understood. It can be said now, however, that the shapes of the distribution curves are facts of some interest, and suggestive of interpretations which can be subjected to further investigation. Moreover, the systematic investigation of the shapes of distributions of various components of body fluid in other invertebrates may afford a valuable supplement to starvation and feeding experiments in the investigation of regulation and its evolution in the blood.

Summary

1. The degree of gonadal development in the purple sea urchin, *Strongylocentrotus purpuratus*, was measured by taking the gonad index, or ratio of volume of gonad to wet weight of urchin, every two weeks for a year (1953–54) in samples of 20 urchins obtained from each of two localities, Pescadero Point on the Monterey Peninsula, and Yankee Point, 10 miles south.

2. For *S. purpuratus* from both places, the average gonad index rose during the fall to a peak twice as high as that reached the previous year, and this peak was maintained at a fairly constant level from January through May.

3. Climatic factors, rather than ecological, are therefore thought to have the greater effect upon gonad growth. A clear-cut correlation was not established for any factor, but ocean temperature is known to have been higher during the winter of 1953–54 than in the previous year.

4. Determination of the gonad index for monthly samples of 10 specimens of the large western sea urchin, *S. franciscanus*, taken from below the tide pools of the Hopkins Marine Station, indicated a single reproductive cycle, but a much shorter breeding season, the peak of which was reached later than that of *S. purpuratus*.

5. Though males were found to have fertile gametes for a greater part of the year than females, no significant difference was found in the reproductive cycles of male and female urchins in either species, as measured by gonad index.

6. The great growth of gonads was not correlated with an increase in NPN (non-protein nitrogen) or RS (reducing sugar) in the body fluid of either species. However, greater mobilization, *i.e.*, transport from gut to gonad, could not be determined by the methods used.

7. No seasonal variation of RS was found in either species. NPN was also invariant in *S. purpuratus*, but showed a marked increase in *S. franciscanus* during the summer.

8. In both species there was a much greater variability of RS than of NPN, suggesting no regulation of RS, but a degree of regulation of NPN. RS was skewly distributed, while NPN was normally distributed in nearly all samples, a fact which may be partially explained by the feeding habits of the animals.

9. Very small quantities of protein nitrogen of the order of 0.5 mg. per cent were found in the filtered body fluids of a few specimens of each species.

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EVIDENCE FOR AN EXOGENOUS CONTRIBUTION TO PERSISTENT DIURNAL AND LUNAR RHYTHMICITY UNDER SO-CALLED CONSTANT CONDITIONS [1]

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Daily rhythmicity which persists in conditions of constant darkness and temperature has been reported for great numbers of animals and plants ranging from the simplest one-celled ones to the most complex multicellular ones. One of the most striking aspects of this rhythmicity is that it has been reported to continue with great precision, retaining its normal phase relationships with respect to the external day-night cycle over long periods of time. Since most of the observations have been made following the placing of animals in darkness at some arbitrarily selected temperature, virtually all of the studies have implied a temperature-independence of the mechanism responsible for maintaining the frequency of the daily cycles over at least a few degrees. A critical study of the temperature relationships of the daily rhythmicity in fiddler crabs (Brown and Webb, 1948) confirmed the temperature-independence of the frequency over the range, 6° to 26° C. At temperatures of 0° to 2° C. the mechanism responsible for regulating the frequency of the color change rhythm in the fiddler crabs was greatly depressed. More recently (Webb, Bennett, Graves and Stephens, 1953), it has been found that there is a daily rhythm in the degree of depression of the frequency of the mechanism at a temperature of 5° C., with only about 56% depression in the morning hours and about 78% depression during the evening hours. More recently, temperature-independence has been reported for the daily rhythm of emergence of the fly, Drosophila (Pittendrigh, 1954).

The persistent daily rhythms of many animals have been shown to continue with their accurately regulated 24-hour cycles even though the phases of the cycles have been shifted experimentally so that they no longer bear their normal relationships to the external day-night cycles. These cycles may be shifted experimentally either by exposing the animals to altered times of light and darkness in 24-hour cycles, or by subjecting them to various periods of refrigeration. The shifted rhythms typically display no tendency to drift back to their normal relationships to the external physical ones.

Persisting primary lunar or tidal cycles have also been reported. The literature on these is surveyed briefly by Brown, Fingerman, Sandeen and Webb (1953), who described a persisting tidal rhythm of color change of the fiddler crab. The crabs, which simultaneously possessed persisting 24-hour cycles, showed in con-

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stant darkness semilunar rhythms which appeared to result from the periodic reinforcement of one of these rhythms by the other. Temperature-independence of the tidal rhythms was demonstrated by Brown, Bennett, Webb and Sandeen (1954). The tidal rhythm, like the daily one, could be shifted by illumination changes and thereafter the rhythm would not drift away from the new setting. In fact, it was shown unequivocally that the phase relationships of the normal tidal cycles were determined by the times of the tides of each local beach, and these would be retained in constant conditions.

It has been assumed by many investigators that the rhythmic changes in constant conditions were regulated by an internal clock capable of measuring very accurately periods of 24 hours. The existence of such an internal clock was finally established definitely by Brown, Webb and Bennett (1955). These investigators found that fiddler crabs carried in darkness from Woods Hole, Massachusetts, to Berkeley, California, (*i.e.*, through 51° of longitude) by airplane continued to possess a daily rhythm of color change essentially in phase with that of crabs left under similar conditions in Woods Hole, as controls. The transported crabs clearly had been able to measure a reasonably precise 24-hour period even during a period when every conceivable factor whose frequency was determined by the rotation of the earth had been extended to a cycle of about 27.3 hours. There was, however, a suggestion from this experiment that the accuracy of this internal clock, under these conditions of uncontrolled temperature, might not be great enough to account for the astounding precision that would be required for the cycles not to get measurably out of their normal phase relationships with the external day-night cycles during several weeks or even months as has been observed frequently.

The research to be described was conducted in order to learn more about the nature of any daily and lunar rhythms in the rate of O_2-consumption with the view that some clue might be forthcoming as to the nature of the means whereby temperature-independence of the frequency of the cycles in organisms might have been achieved.

Materials and Methods

For the experiments to be described, the salamander, *Triturus viridescens,* and two species of fiddler crabs, *Uca pugnax* and *Uca pugilator,* were used. The experiments with *Triturus* were performed in Evanston, Illinois. The salamanders were shipped to Evanston from western Massachusetts where they had been collected. The experiments with *Uca* were performed at Woods Hole, Massachusetts, using crabs which were collected at Chapoquoit, on Buzzards Bay about 6 miles north of Woods Hole.

The crabs, each weighing about 3 grams, and *Triturus,* similarly weighing about 3 grams each, were placed in individual respirometers and their rates of O_2-consumption measured continuously in automatic, recording respirometers (Brown, 1954). This type of respirometer permits the animals to be maintained in essentially constant O_2-tension, and to be subjected continuously to the natural fluctuations in barometric pressure throughout the course of the experiments.

In its essentials the respirometer comprised a 50-ml. Soxhlet distilling flask possessing a rubber stopper bored part way through with a cork-borer. The

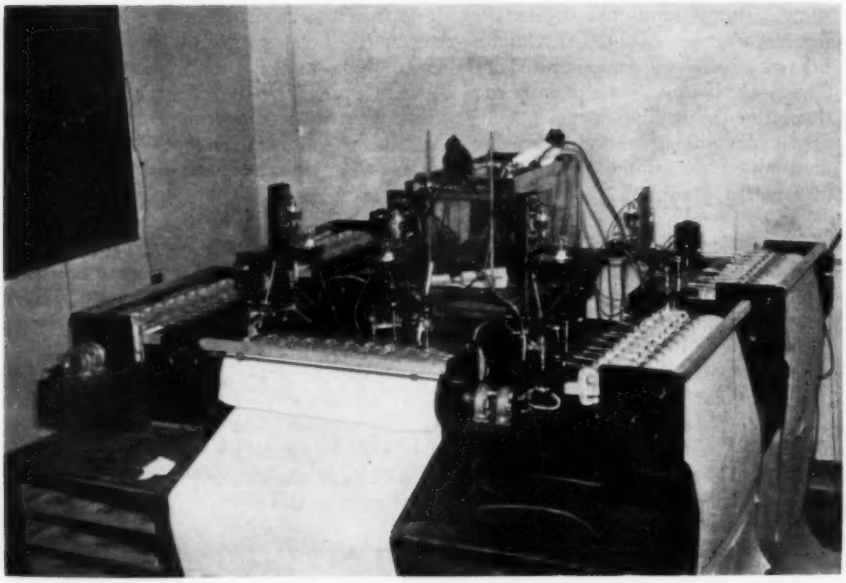

FIGURE 1. The arrangement of the baths, temperature-regulating equipment, and the recording respirometers operating from June 19 through August 30, 1954, at Woods Hole, Mass.

stopper was penetrated by a 27-gauge hypodermic needle with its point directed upwards through the stopper. To the lower side of the stopper were attached vessels for the CO_2-absorbent (KOH) and the ammonia-absorbent ($CuCl_2$) which were suspended into the flask. To the upper side of the stopper was sealed a plastic (Saran) collapsible sack which was, for all practical purposes, impermeable to O_2. In operation the animal was placed in the flask in a small amount of water; the sack was filled with oxygen; the vessels were filled with the absorbents, and the stopper set tightly in place. This ensemble, constituting a diver, was provided with just enough attached lead weights to sink it in water. It was then suspended by a fine wire in a constant temperature bath. The fine wire was attached to a very delicate spring scale with ink-writing points of such a character that the weight of the diver would be recorded continuously upon a moving strip of paper. The paper was drawn over a drum revolved by a telechron clock motor (Fig. 1).

It is evident that as the animals consumed oxygen, oxygen from the plastic sack would flow down the needle at a rate just adequate to compensate for that consumed by the organisms. For each milliliter of oxygen consumed, the diver would be expected to gain in weight by one gram. Since the sack had a capacity of about 50 ml., this was an adequate supply of oxygen to maintain the animals for upwards of a week without attention. In practice, however, the divers were opened at 3- to 4-day intervals, the O_2 and absorbents refreshed, and occasionally new animals were substituted at these times for those that had been in the respirometers for several

days. Only extremely rarely did an animal die in a respirometer; in these cases the whole record for that organism, from the time the respirometers were last refreshed, was discarded.

The constant temperature baths in which the divers were suspended were maintained at 19.7° C. for the experiments in Evanston with *Triturus*, and 19.4° and 19.9° for the two baths which were used at Woods Hole for the experiments with *Uca*. In order to shield the divers from the agitated water of the bath, an aluminum container was submerged in the main bath, and it was in this aluminum container that the divers were suspended.

In all the experiments the animals were maintained in rooms protected from outdoor illumination. Incandescent sources gave a constant illumination of less than one ft. c. at the surface of the baths. The baths and aluminum container were painted flat black on their inner walls. Hence the organisms themselves were subjected to a much lower constant illumination which was not measured.

The experiments with *Triturus* were performed between May 12 and June 9, 1954. During this 29-day period there were continuously in operation eight respirometers containing *Triturus* and one respirometer treated in exactly the same manner except that no animal was added which was maintained as a control.

The studies of *Uca* were carried on without break between June 19 and August 31, 1954. Four crabs of each of the two species were run in each of two baths. In addition to the eight respirometers containing crabs, there was in each bath a diver lacking only the animal, run as a control.

In some earlier studies on O_2-consumption in snails and fiddler crabs (Sandeen, Stephens and Brown, 1954; Brown, Bennett and Webb, 1954) it had been assumed that the blank divers could be used as a proper means of correction for the concurrent fluctuations in barometric pressure. A more critical evaluation of the validity of this procedure has now clearly indicated that it is not a satisfactory method. In Figure 2 are depicted the variations in apparent mean respiration of eight *Uca pugnax*, over an arbitrarily selected 4-day period uncorrected for barometric-pressure effects. Superimposed upon this record, and plotted on the same scale are (1) the mean variations observed in five blank divers run concur-

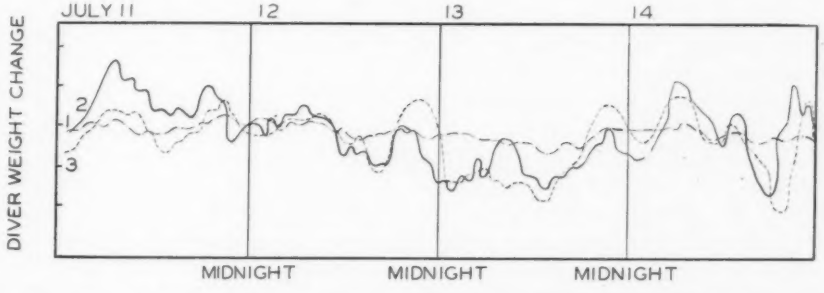

FIGURE 2. Plotted on the same scales are seen 1) the average hourly weight changes in the blank, control divers, 2) the average hourly weight changes for the crab-containing divers, and 3) the theoretically expected hourly changes in the blank divers on the basis of the actual barometric pressure changes, during an arbitrarily selected 4-day period.

rently, and (2) the expected variations in the blank divers calculated on the basis of the simultaneously recorded changes in actual barometric pressure. An analysis of 29 days of blank-diver data indicated that the blanks were responding, on the average, only about 27% of the theoretically expected extent. This error was finally traced to the mechanics of the spring scale. In order to decrease the sensitivity of the spring scale so that 3 to 4 days of uninterrupted recording of respiration of the animals could be obtained the spring scale had been adjusted so that the fluctuations in barometric pressure produced only relatively small movements in the long writing arm. In view of the virtual freedom of the blank divers from gaseous escape, the random fluctuations in barometric pressure produced only very small right and left excursions of the pen about a mean. Therefore, a large fraction of the expected range of pen variation was absorbed in a small freedom of movement in the pivotal bearings of the pen. The reality of this error was confirmed later by attaching blank divers to more sensitive scales. Under these latter circumstances the blanks approached more closely the changes expected in response to the actual pressure changes.

On the other hand, it can be seen that the respirometers containing the animals appeared to be behaving like more nearly perfect barographs. The total range of variation and the temporal relationships of most of the maxima and minima suggest this. Here it is evident that freedom of movement in the pivotal bearing of the scale writing arm was not of any influence. Due to a continuous utilization of O_2 by the animals, the pen was under essentially continuous, unidirectional tension. The pressure changes were translated into the record simply as spurious increases and decreases in rates of O_2-consumption.

In further confirmation of the assumption that the divers containing animals were actually behaving as essentially perfect barographs is a comparison of the average form of the daily variation in rates of gain in weight by the divers containing animals for 29 days, on the one hand, and the average form of the daily variation expected for blank divers on the basis of the measured variations in barometric pressure for the same 29-day period. This is seen for a 29-day period with the crabs, Figure 3A, and for the 29-day experimental period with *Triturus*, Figure 3B. It is seen clearly that not only are the phase relations essentially the same for the animal-containing respirometers and the blanks, but also the amplitude of the variation is closely similar. If the correction had been either 15 or 20% larger or smaller, rhythms simply correlated with barometric pressure would have been evident for all three species.

In the computations of the effect of barometric pressure, a diver volume of 100 ml. was always assumed and the corrections were calculated on the basis of an initial pressure of 30.00 inches Hg. Less than a 2% error was introduced in using this arbitrarily selected pressure instead of the actual barometric pressure. Assays of the divers revealed a mean volume of about 100 ml. for the 3- to 4-day respirometer runs. During this period there was a gradual volume reduction from about 110 ml. to about 90 ml. Therefore, about a 10% error was introduced through the use of this mean volume of 100 ml. The gradual drop in volume of the respirometers as the oxygen was utilized over 3 or 4 days would not contribute any rhythmic component of the frequencies being investigated. The general validity of this method is borne out by the gross good fit of the observed respirom-

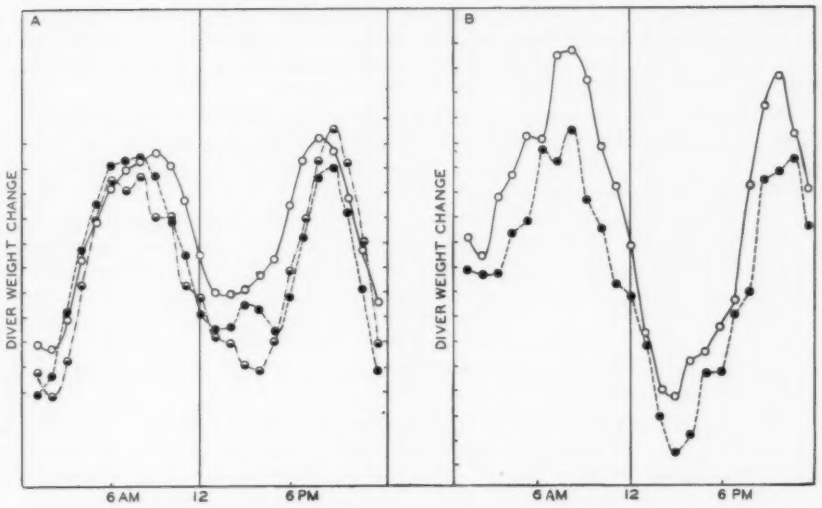

FIGURE 3. A. The mean daily variation in weight changes for a 29-day period of 1) divers containing *Uca pugnax* (open circles) and 2) divers containing *Uca pugilator* (solid circles) and 3) the theoretically expected 29-day, mean daily variation in the divers expected on the basis of the actual barometric pressure change (half open circles). B. The mean daily variation for 29 days of data with 1) divers containing *Triturus* (open circles) and 2) the mean daily diver variation expected on the basis of the pressure changes for the same period (solid circles). All are plotted on the same scale.

eter fluctuations and the calculated fluctuations based upon the concurrent pressure changes (Figs. 2 and 3).

However, since there is a clear mean daily rhythm of barometric pressure with an amplitude of the order of 0.05 inches Hg, it would be expected that there would be a mean daily rhythm imposed upon the volume of the respirometers and hence be reflected in the data corrected for the pressure on the basis of an initial pressure of 30.00 inches Hg used here. At those times of day when the barometric pressure is above the daily mean the corrections used would be too large, and for those times when the pressure was below the average, the corrections would be too small. The cyclic influences of the barometric pressure would be expected to be very slightly amplified, as a result of the correlated slight variation of the diver depth in the water bath and also through the operation of Henry's Law with regard to the water in the bottom of the respirometer chamber. A slight error is also introduced in correcting the volume as for a dry gas.

However, when one compares the form of the daily variation expected solely on the basis of the influence of the mean daily variations in barometric pressure on the respirometers, one finds in no case the form of the mean daily cycle of O_2-consumption to correspond. The rhythms appear to possess distinct species-specificity.

In the determination of the mean hourly correlation between the barometric pressure rate and direction of change and rate of the concurrent O_2-consumption,

the values used in the calculation of each point included, indiscriminately, both overcorrected and undercorrected data due to any imposed barometric-pressure daily cycles. Therefore, the good correlations which were found are considered to be true biological responses.

It should also be pointed out that in setting up afresh the respirometers at three- to four-day intervals, the respirometers were closed a number of times in each 15-day or 30-day series, and in a random manner with respect to the actual barometric pressures at the times of change.

As a consequence of the foregoing considerations, the hourly respirometer data for the salamanders and crabs were corrected for the calculated influences of barometric pressure changes on the divers, upon the basis of a mean diver-volume of 100 ml. and of a uniform barometric pressure of 30.00 inches Hg.

Results and Their Analysis

The rates of O_2-consumption for *Triturus* for each hour of the day for the 29-day period, May 12 through June 9, 1954, were determined and averages obtained for the eight animals which were studied concurrently. The rate of respiration during the 29-day period varied through a range of several hundred per cent from lowest to highest values. There was no regular pattern of daily variation, or overt daily rhythm. Superficially the day by day variation appeared to be random.

The rates of respiration for *Uca pugilator* for every hour of the period June 19 through August 30, 1954 were determined as were also those for *Uca pugnax* for the same period. As with *Triturus*, neither of the crabs exhibited any regular variations in rates of respiration from hour to hour or from day to day. There was not an overt daily rhythm of respiration. The variation during the period of observation yielded a range of the order of several hundred per cent from lowest to highest values, and appeared to be random in character.

The mean value of O_2-consumption for *Triturus* for the 29-day period was 28.12 ± 0.57. The mean value for *U. pugnax* for the 29-day period from July 27 through August 24 was 21.19 ± 0.43. The mean value for *U. pugilator* for the same period was 32.55 ± 0.53. If these values are multiplied by the conversion factor, 1.6, they become ml./Kg./hr. There seemed to be no evident correlation in forms of the day by day variations between *Triturus*, which was studied in May and June on the one hand, and the fiddler crabs which were studied in July and August, on the other, even when the days of data were synchronized as nearly as possible relative to the phases of the moon. Lack of correlation was confirmed when a determination of the coefficient of correlation of the hourly data for *U. pugnax* and *Triturus* with data brought as closely as possible into phase relative to sun and moon revealed a value of 0.0362 ± 0.038.

On the other hand, there appeared on superficial examination to be a significant correlation between the variations in the two species of fiddler crabs which were studied concurrently. A determination of the coefficient of correlation for the hourly values for the two species for the 29-day period July 27 through August 24 yielded a value of 0.401 ± 0.032. With the coefficient of correlation more than 12 times as large as its standard error, the existence of a positive correlation is beyond all reasonable doubt.

The random character of the hour by hour variations was emphasized when one determined the coefficient of correlation for the hourly values for *Uca pugnax* on

day n with the values for *Uca pugilator* for day $n + 1$. An analysis of 29 days of data (June 19 through July 18) for such data dislocated by one day showed there to be no correlation (0.0117 ± 0.037). A low degree of correlation was seen when the values for day n were correlated with the values for the day $n + 1$ for a period of a month for the same species. This last, for *Uca pugnax*, provided a coefficient of correlation of 0.176 ± 0.034 and indicated a small tendency of the crabs to repeat on the next day the pattern exhibited on any given day.

On the other hand, when one attempted to correlate the hourly data for *Uca pugnax* and *pugilator* for one synodic month with the same species for the same hours of the days of the succeeding month, there was found for *pugnax* a value of 0.103 ± 0.032, and for *pugilator*, 0.007 ± 0.038. There is a suggestion of a correlation, even though a small one, in the hourly values of O_2-consumption for *pugnax* through days possessing comparable lunar relations. A similar suggestion was absent for *pugilator*.

In earlier work with fiddler crab rhythms of color change (Brown, Fingerman, Sandeen and Webb, 1953) and in respiration (Brown, Bennett and Webb, 1954) it was possible to resolve the variations into a complex of rhythms of different frequencies. These included ones of daily, primary lunar, semi-monthly and possibly monthly cycles. In order to determine if similar rhythms were present in these organisms using the more extensive and more critically corrected data, the data were first analyzed in such a manner that any existing daily cycle would be made evident. Since in a synodic monthly period every phase of a primary lunar

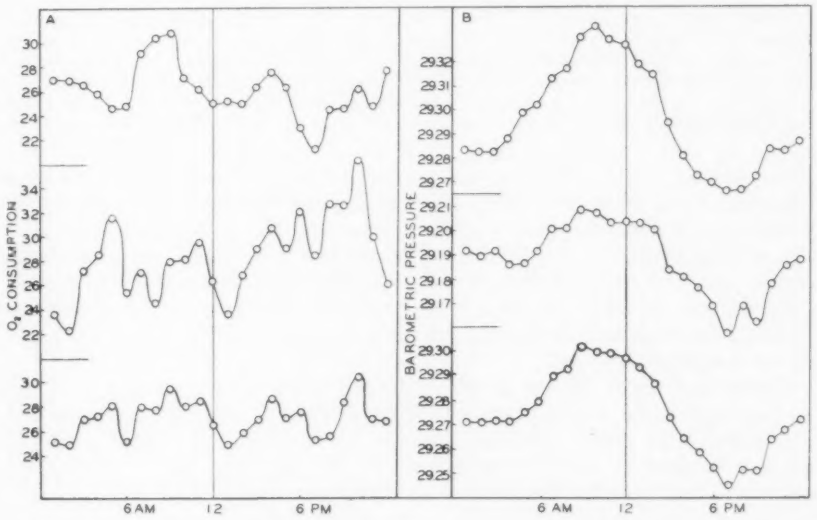

FIGURE 4. A. The mean daily variation in oxygen consumption for *Triturus* for 1) a 15-day period centered on full moon, 2) a 15-day period centered on new moon, and 3) a 29-day period. B. 1, 2, 3. The mean daily variations in barometric pressure for the two 15-day and 29-day periods in A, respectively.

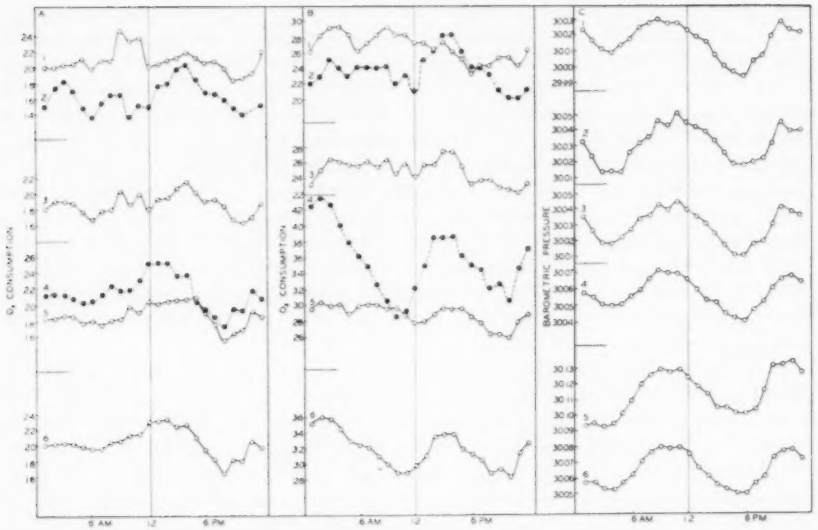

FIGURE 5. A. 1 and 5) The mean daily variation in oxygen consumption in *Uca pugnax*, for two consecutive new moon-centered 15-day periods. 2 and 4) The mean daily variation in oxygen consumption for two consecutive full moon-centered 15-day periods. 3 and 6) Two consecutive 29-day, mean daily variations in oxygen consumption. B. Comparable mean daily variations in oxygen consumption for the same periods as in A, but now for *Uca pugilator*. C. Mean daily barometric pressure variations for 1) the new moon- and 2) full moon-centered 15-day periods of the first month, and 3) the 29-day mean variation of the first month; 4) the new moon- and 5) the full moon-centered 15-day periods of the second month and 6) the 29-day mean variation of the second month.

cycle is present in the values for every hour of the day, the forms of the mean daily cycles were determined using 29 or 30 days of continuous data. In Figure 4A is found the mean daily variation for *Triturus* for the 29-day period, the mean daily cycle for each of two semilunar periods, and in Figure 4B the mean barometric pressure cycles for the same three periods. In Figure 5A, B and C are found comparable 15-day and 29-day mean daily cycles for *Uca pugnax* and *U. pugilator*, and the concurrent mean barometric pressure cycles for the periods June 23–July 21 and July 22–August 21.

The 29-day mean daily cycle of respiration for *Triturus* appears to possess four maxima, one in each of the four quarters of the day. The 29-day mean daily cycles for the two species of crabs appear to be rather similar to one another in their general form. There is a tendency for maxima to occur about 2 to 3 A.M. and about 2 to 4 P.M., with the lowest rate of the day occurring in the evening.

The daily rhythms for fortnights were calculated for periods with either new moon or full moon centered in the period. During 15-day periods from last quarter to first quarter the moon is above the horizon at noon and during the 15-day periods from first quarter to last quarter it is below the horizon at noon.

It is readily seen in the mean 15-day cycles that in every instance the form of the daily cycle differs between the two fortnights. In the fortnight straddling the full moon for *Triturus,* the lowest values of the day are in the late afternoon or early evening; the reverse is true of the fortnight straddling the new moon, the lowest values occurring before noon. For both species of fiddler crabs the mean daily cycle for the two fortnights straddling new moon exhibit only little change in rate through most of the day but show a clear minimal rate in the evening. For the fortnights straddling full moon, on the other hand, a much more prominent 2 to 4 P.M. peak in respiration becomes evident. In brief, there is clear suggestion in these fortnightly mean daily cycles of the existence of a persistent monthly cycle. The mean daily variations in barometric pressure also vary in every instance between the two 15-day periods. The morning rise in pressure is always greater for the fortnight straddling a full moon than for the fortnight straddling a new moon.

In order to determine the nature of any rhythmic differences through the month between the morning and afternoon values of respiration, the rate for the hours 5, 6, and 7 A.M., on the one hand, and 5, 6, and 7 P.M., on the other, were compared with one another and with both the mean hourly averages for the whole days and the barometric pressure means for the early morning and early evening hours. All are illustrated in Figure 6, 6A for the crabs and 6B for *Triturus.* In Figure 6A it is seen that there is a general tendency for the respiration of the crabs at the two times of day to fluctuate in the same manner as the daily mean values. While there is some correlation between the two species, there are also some conspicuous differences. There is not much evident correlation with barometric-pressure changes over the same period. A casual inspection suggests, however, that in the initial stages of a period of gradual increase in barometric pressure, there is a tendency for the O_2-consumption to decrease. Similarly, in the initial stages of a period of gradual fall in pressure, there tends to be an elevation in rate of respiration. This correlation becomes less, and it appears sometimes even to reverse itself after two or three days of a continuing rise or fall in barometric pressure. It is also to be observed that just as the morning values of barometric pressure tend to be higher than the afternoon ones during days of gradually declining pressure, and to be higher than the morning ones during days of gradually rising pressure, so do also the rates of respiration show comparable shifts in position, though not correlated with the barometric pressure itself.

In Figure 6B it is seen that what has been said for the crabs seems, in general, to hold for the salamander. Here, for the 5–6–7 P.M. period of the day there would appear to be almost an inverse correlation between pressure and the rate of O_2-consumption. This is less evident for the mean hourly values for the days, and virtually absent for the 5–6–7 A.M. values of respiration.

There is a suggestion for all three species of a tendency for the 5–6–7 P.M. values to possess maxima at about two-week intervals. The maxima tend to occur for these hours at the times of first and third quarters of the moon for the crabs and new and full moon for the salamander.

The data for the three species were next analyzed to learn what might be the general form of any existing primary lunar cycle of respiration. In order to differentiate a primary lunar cycle it was necessary to randomize the daily cycle. To accomplish this it was necessary to utilize a period of 29 or 30 days of continuous

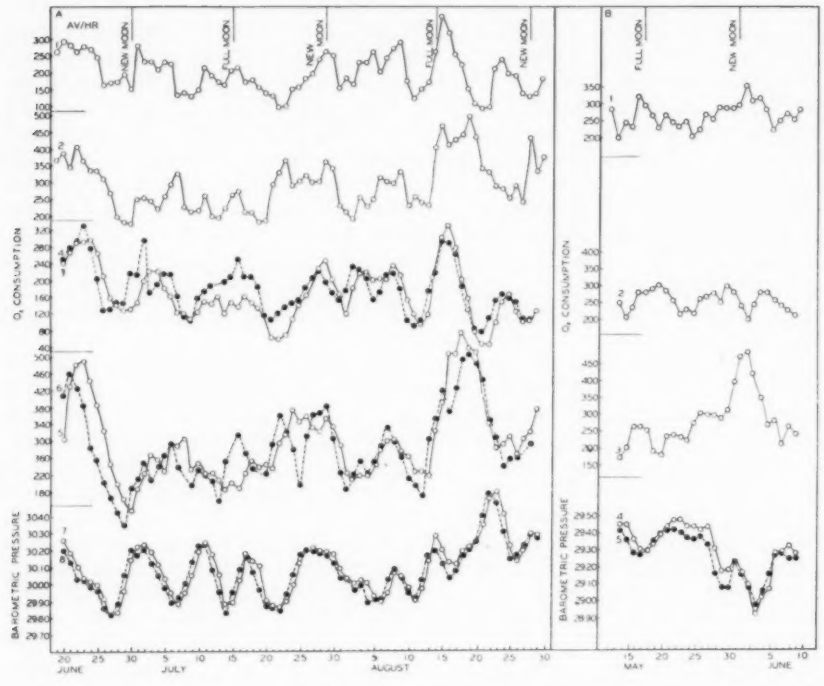

FIGURE 6. A. 1 and 2) Fluctuations in the mean daily rates of O_2-consumption of *Uca pugnax* and *Uca pugilator*, respectively, with time. 3 and 4) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. mean rates of O_2-consumption, respectively, of *Uca pugnax*. 5 and 6) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. mean rates, respectively, of O_2-consumption in *Uca pugilator*. 7 and 8) Fluctuations in the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M., respectively, barometric pressures. B. 1) Fluctuations in the mean daily rate of O_2-consumption of *Triturus* with time. 2 and 3) Fluctuations in the 3-day, sliding averages of O_2-consumption of *Triturus* for the 5–6–7 A.M. and 5–6–7 P.M. hours, respectively. 4 and 5) Fluctuations in, respectively, the 3-day, sliding averages of the 5–6–7 A.M. and 5–6–7 P.M. values for barometric pressure.

data for each of the organisms. The 24 hourly values for the first day were arranged in a horizontal row. The data for the second day were placed, hour by hour, beneath those of the first day. The hourly values for the third, fourth, fifth, and sixth days were each shifted one hour to the left of the daily values lying just above each of them. Thus, the column under the noon values for the first two days would have the 1 P.M. value for the third day, the 2 P.M. value for the fourth, the 3 P.M. value for the fifth, and the 4 P.M. value for the sixth. The remaining 23 to 24 days of data were used in a comparable manner, continuing the shifting of the data to the left at the rate of five hours for every six days, or in other words, at the average rate of 50 minutes a day. After the 29 days of data have been used fully it is evident that a primary solar cycle has just scanned the 24 vertical columns be-

neath the values for the first day. The triangular block of data which no longer lies underneath the first day of data is then moved as a block to fill in the triangular void beneath the initial day. There are now 24 columns of 29 or 30 values in which any cycles of primary lunar frequency are now closely synchronized and there has been a neutralization of the daily cycle.

In Figure 7A are to be found mean primary lunar cycles for *Triturus*, and for the two species of crabs. In Figure 7B are the corresponding cycles of barometric pressure change calculated in the same manner from barographic data. It is evident that all three animals possess a cycle of variation in O_2-consumption of lunar-day length. In the figure all of the graphs have been synchronized with respect to the actual lunar phases, and the times of lunar zenith and nadir relative to them are indicated. Although the forms of the mean cycles vary not only with the species but to some extent even with the particular months for which they were determined, every one of them also exhibits an approximate lunar tidal cycle of about 12½ hours, with a minimum occurring 2 to 6 hours before lunar zenith and about the same interval before nadir.

The mean variations of lunar frequency for the barometric pressure appear somewhat irregular, although there is some suggestion of two tidal cycles in the lunar day in these data as well. The uniformity in the lunar cycles of the three organisms, however, is far more evident and similar than are the cycles of the barometric pressure.

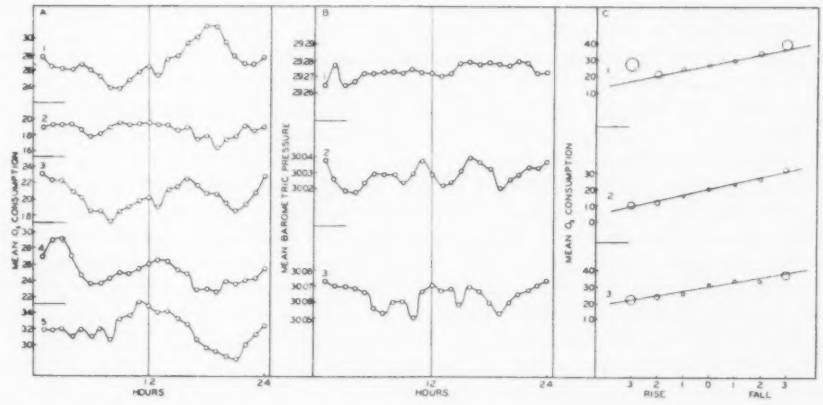

FIGURE 7. A. 1). The mean, 29-day, primary lunar variation of O_2-consumption in *Triturus*. 2 and 3) Two consecutive mean 29-day primary lunar cycles for *Uca pugnax*, and 4 and 5) two consecutive 29-day primary lunar cycles for *Uca pugilator*. Zenith is at 12 hours, nadir at 24. B. The mean variations in barometric pressure of primary lunar frequencies for 1) the 29-day period of the observations on *Triturus* and 2 and 3) the two consecutive, 29-day periods of study of the lunar cycles of *U. pugnax* and *U. pugilator*. C. 1) The relationship between the mean hourly rate of O_2-consumption in *Triturus* and the concurrent rate and direction of barometric pressure change, using all the hourly data of the 29-day period. 2) The same for *U. pugnax* using all the data of a two-month period. 3) The same for *U. pugilator* using all the data of a two-month period. The standard errors of the means are indicated by the diameters of the circles.

Since there was found to be a rather good positive correlation between the rates of O_2-consumption, hour by hour, for the two crabs which were studied concurrently, and either much less or no correlation when the daily values were correlated with the data just one day out of phase, it seemed suggestive that the rhythms of O_2-consumption were being regulated with respect to their frequency, at least, by hour-by-hour variations in some factor of the external physical environment. Consequently, the coefficients of correlation were determined for the hourly values of respiration for the three animals and the concurrent rate of change of barometric pressure ranging continuously from the maximum hourly fall to maximum hourly rise. The data for all three species gave highly significant negative coefficients. The value for *Triturus* was -0.457 ± 0.031; for *Uca pugilator* it was -0.375 ± 0.032 for one month and -0.320 ± 0.034 for the next; and for *Uca pugnax* it was -0.510 ± 0.027 for one month and -0.568 ± 0.027 for the next. In other words the rate of O_2-consumption appeared to be a clear function of the concurrent rate of pressure change; the greater the rate of pressure drop, the higher the rate of respiration, and the greater the rate of pressure rise, the lower the rate of respiration. Furthermore, in comparing the values for the two months for the two species of crabs, there would appear to be a species difference in the amount of the correlation.

Although the correlations are all quite high and unquestionably significant relative to the concurrent rates of barometric pressure change, when one determines the correlation with the same hourly rates of barometric pressure change for the preceding day (respiration, day n, vs. barometric pressure changes, day $n-1$), values for *Uca pugnax* were -0.068 ± 0.037 for the period June 27–July 26, and -0.059 ± 0.037 for the period July 28–August 26. This appears to be a very low negative correlation, but its statistical significance is questionable. On the other hand, when a correlation was made for the same species with rates of barometric pressure change for the succeeding day (respiration, day n, vs. barometric pressure changes, day $n+1$), a most unexpected result was obtained. For the period June 27–July 26 the coefficient was found to be -0.135 ± 0.037; for the period July 28–August 26 the value was -0.142 ± 0.037. Both of these values are of the order of twice the size of the correlation with the preceding day and each is statistically significant. It was obvious that the correlation of the barometric pressure change for the two pairs of days (n vs. $n-1$ and n vs. $n+1$) of the preceding periods would be essentially the same. When the hourly rates of barometric pressure on day n were correlated with the hourly rates on day $n+1$ for the same period a value of 0.176 ± 0.036 was obtained. For barometric pressure changes, day n, correlated with barometric pressure changes, day $n+2$, for the same period, it was 0.136 ± 0.037.

On the other hand, O_2-consumption in *Uca pugilator* for the hours of day n, whether correlated with the pressure changes during day $n+1$ or day $n-1$, gave equally low values, 0.002 ± 0.037 and 0.029 ± 0.037, respectively. If the difference seen for *Uca pugnax* should prove to be other than fortuitous and even be found for some other species, a possible conclusion would be that the crabs were responding to some environmental factor which itself in some manner is associated with an influence on the character of change in pressure for the next 24 hours or so.

The relationship between the rate and direction of barometric pressure change and the mean rate of respiration in the three species is illustrated in Figure 7C. Here are indicated both the mean rates and their standard errors. O_2-consumption

values correlated with barometric pressure rates of change beyond .03 inches Hg/hr. were not plotted in these figures inasmuch as so few instances were available that their significance could not be established. For *Triturus*, a rise or fall of 0.01 inches Hg/hr. produces about a 12% change in respiration; for *Uca pugnax* a similar rate of change is correlated with about an 18% change, and for *Uca pugilator* with about a 9% change.

There appeared also to be a correlation between the absolute barometric pressure and the rate of respiration as was suggested in Figure 5A for the fiddler crabs. A calculation of the coefficient of correlation based upon the hourly values for *Uca pugnax* for the two-month period, June 27–August 25, and the concurrent barometric pressures provided a value of 0.057 ± 0.026. For *Uca pugilator* for the period, June 27–July 26, the corresponding value was 0.192 ± 0.036; and for *Triturus* for the period, May 12–June 9, it was -0.149 ± 0.038. The last negative correlation had been suggested in Figure 5B. It is evident from these values that although significant correlations with barometric pressure are present for at least *Uca pugilator* and *Triturus*, these are of substantially lower degree than the correlations with the concurrent rates of barometric pressure change.

In less extensive experiments in which only 5 or 6 days of data were obtained a correlation was found between the rate of O_2-consumption and concurrent rate and direction of barometric pressure change for the three other invertebrates. For the sipunculid, *Phascolosoma gouldii*, the coefficient was -0.480 ± 0.064. For the sea-cucumber, *Thyone briareus*, it was -0.472 ± 0.069. The mud snail, *Nassa obsoleta*, gave a coefficient of -0.264 ± 0.083.

Discussion

It would appear from this study that fiddler crabs and *Triturus* have daily, primary lunar and monthly rhythms of O_2-consumption which are not overt ones but which resemble the external rhythms in the physical environment in being statistical. Quite similar rhythms have also been found for O_2-consumption in three plants, the potato, carrot, and a seaweed, *Fucus* (Brown, Freeland and Ralph, 1955.) The daily and lunar rhythms in the plants resemble those of the animals reported here, even in some detail. The potato and *Fucus*, furthermore, showed essentially the same character of correlation with barometric pressure as found for these animals. The carrot, on the other hand, showed a reduction in rate of O_2-consumption whenever the barometric pressure was either rising or falling, with the degree of reduction linearly related to the rate of change.

These cycles of O_2-consumption show a good similarity with the rhythms of opening and closing of the shells of the oyster under constant conditions (Brown, 1954b). The activity of the oysters was recorded continuously for 45 days by simple, mechanical, automatic recorders. The oysters similarly showed no overt daily or tidal cycles, but only statistical ones. The forms of the mean daily cycles obtained with the oysters, and the variations of the daily cycles with the two halves of a synodic month, resembled those found in the work reported here on O_2-consumption in all three species. The oysters tended to be open more when barometric pressure was falling and to be open less when barometric pressure was rising. This general confirmation of the forms of the daily and lunar rhythms found in O_2-consumption obtained with the automatic-recording respirometers by results using

simple, mechanically-recorded activity in the oyster, gives further support to the method of barometric pressure correction which was used in this work.

Any external factor, either barometric pressure or some factor which shows some degree of correlation with pressure which influences the rate of O_2-consumption in the organisms in the respirometers appears, therefore, also to influence the average number of minutes per hour that groups of oysters are open. It seems reasonable to presume that any factors which increase the rate of O_2-consumption in the oysters would, in consequence, cause the oysters to remain open a greater percentage of the time, and on this basis the results become consistent in the sign of the relationships.

These rhythms which are imposed upon the animals by the rhythmic external factor or factors appear not to be overt ones. Barometric pressure changes include a very large random component. Furthermore, for these variations to be resolved into rhythms of a clear daily character requires the integration of several days of barometric pressure change data. It is also true that the pattern of the daily changes in overt rhythms such as those of color change in the crabs (Brown, Fingerman, Sandeen and Webb, 1953), retinal pigment migration in shrimp (Webb and Brown, 1952), are usually quite different from that of the basic metabolic rhythm and, for that matter, from one another. There is some reason to believe that the overt rhythms are directly regulated by an internal clock, and that it is this internal clock which is reset by light and temperature. The imposed rhythm seems to be unmodified when the internal clock is reset. In two experiments with *Uca pugnax* in which the daily cycle of color change was essentially inverted by 12-hour periods of chilling, the correlation of the hourly values of O_2-consumption with those of unshifted controls gave coefficients of 0.389 ± 0.035 and 0.395 ± 0.031.

The evidence suggests, therefore, that the imposed and the internally regulated 24-hour cycles may come to bear any phase relations one to the other and then have no tendency to drift to any other relationships.

Although we still know little or nothing about any functional interrelations between the internal and the imposed daily rhythms, it is tempting to speculate that the imposed one in some manner contributes to the observed temperature-independence of the internal clock, at least over extended periods. A reasonably accurate daily internal clock would seem also to be a necessity in the resolution of the phases of the external rhythms which become evident only when several days of data are averaged. The daily clocks of the animals may well be a consequence of the cooperation of these two rhythms. Suggesting this is the often-observed variation in the times that the animals go into the night phase in their overt daily rhythm of color change. The variation about a mean may be more than a half hour from day to day, and yet there is no statistical drift in constant darkness even over months. This would find a reasonable explanation in terms of the internal clock being reset continuously by the rhythms of those factors which are responsible for the imposed clock.

This work provides no answer to the question as to what is the nature of the varying external force which is responsible for the imposed daily rhythm of O_2-consumption. Since the response correlated with the rate of change in barometric pressure is greater than the response correlated with the absolute level of pressure it appears to be a type of force to which there is some accommodation by the organism. Also the relatively low hourly correlation may be in part because even in

superficially good correlations, *e.g.*, Triturus 5–6–7 P.M. the respiratory changes often anticipate barometric pressure changes by one or two days.

The force involved could be barometric pressure itself, or it could be any of a number of other factors which show some degree themselves of correlation with barometric pressure changes. One such other factor is cosmic radiation (Barnothy and Forro, 1939). It has been shown by Brown, Bennett and Ralph (1954), that fiddler crabs exhibit a measurable response in the state of their pigmentary system to alterations in the intensity of cosmic-ray showers obtained by placing lead plates over the animals. Had there been no correlation of the hourly changes in O_2-consumption with barometric pressure changes, then it would have been reasonably clear that such radiation was not normally involved in the regulation of the metabolic rhythms. The correlation with barometric pressure that has been found in this study leaves a role of such radiation as a distinct possibility, though quite obviously it does not, on the other hand, provide any positive evidence for such a role.

There is good likelihood, judging from the known simultaneous influences of such forces as light, temperature and tactile stimuli, that if these organisms possess the capacity to respond to one type of these relatively low-energy, or diffuse, types of environmental stimuli such as are implied by these results, they also possess the capacity to respond to a complex of them. Supporting such a multiple-factor view is the fact that the forms of the rhythms and their monthly variations appear to correlate to some extent with barometric pressure, but at the same time have large significant variations at some times of day and month that show little indication of any correlation with pressure.

Summary

1. Persistent rhythms of primary solar and primary lunar frequencies are described for O_2-consumption of two species of fiddler crabs, *Uca pugnax* and *Uca pugilator*, and for the salamander, *Triturus viridescens*.

2. These rhythms appear to be statistical rather than overt ones. The forms of the rhythms become apparent only upon the averaging of several days of data.

3. The form of the daily variation in O_2-consumption shows a monthly variation, the form of the rhythm for a two-week period straddling a new moon being different from the form for a two-week period straddling a full moon.

4. The hourly values in the rate of respiration show a significant correlation with the concurrent rate of barometric pressure change. In all three species, the rate of O_2-consumption increases in a direct relationship with the concurrent rate of barometric pressure fall and decreases in a direct relationship with the rate of pressure rise.

5. There appears, in some instances, to be a correlation with the absolute concurrent barometric pressure, but this is distinctly less. The correlation was a positive one for the two species of fiddler crabs and a negative one for the salamander.

6. It was demonstrated that O_2-consumption in all three species of animals displayed, therefore, imposed daily rhythms and in all probability, also imposed lunar rhythms.

7. The striking similarity of the forms and phase relations of these imposed daily and lunar rhythms of O_2-consumption to ones which are being described elsewhere for three species of plants, the alga, *Fucus*, and the carrot and potato, is

pointed out. The rhythms are also compared with very similar statistical solar and lunar rhythms of opening and closing of the shells recently described for the oyster.

8. There is a brief discussion of the environmental forces which might be involved, and speculation as to possible relationships between these imposed rhythms and the demonstrated endogenous rhythms in producing the temperature-independent overt rhythms observed in many processes in animals.

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PERSISTENT DAILY AND TIDAL RHYTHMS OF COLOR CHANGE IN CALLINECTES SAPIDUS[1]

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The phenomenon of persistent biological rhythms has been reviewed by Welsh (1938), Park (1940), Kleitman (1949), Webb (1950), and Brown, Fingerman, Sandeen and Webb (1953). These investigators have described diurnal rhythms with a frequency of 24.0 hours, tidal rhythms with a frequency of 12.4 hours or 14.8 days, lunar rhythms with a frequency of 29.5 days, and annual rhythms.

The melanophore pigment of the fiddler crab, *Uca*, shows a striking diurnal rhythm of migration. In its natural environment *Uca* is light by night and dark by day. This rhythm persists in constant darkness in the laboratory for at least several weeks without loss of synchrony with solar day-night or decrease in amplitude (Brown and Webb, 1948). The frequency of this rhythm is not altered by temperatures between 6° and 26° C. It has been demonstrated, however, to be inhibited at temperatures below 6° C.

Brown, Fingerman, Sandeen and Webb (1953) described a persistent tidal rhythm of the pigment in the melanophores of *Uca pugnax* and confirmed the persistent diurnal rhythmicity of this pigment. This tidal rhythm is superimposed upon the diurnal rhythm and leads to a third type, a semilunar rhythm, in which the diurnal and tidal rhythms are in the same phases relative to one another only once each 14.8 days. The phases of the tidal rhythm of pigment migration bear a definite relationship to the time of low tide in the vicinity of the Marine Biological Laboratory, Woods Hole, Massachusetts where the *Uca* were collected. In the Woods Hole area the normal tidal situation is semidiurnal, two low tides and two high tides during each lunar day of 24.8 hours.

The Gulf of Mexico and its adjacent waters exhibit a primarily diurnal tidal situation of one low tide and one high tide per lunar day. This tidal picture provides an excellent contrast with the situation at Woods Hole. This investigation was undertaken to observe the phenomena of persistent daily and tidal rhythms of color change in a species living under the influence of diurnal tides.

Materials and Methods

The animals observed were immature blue crabs, *Callinectes sapidus*, of 10 to 40 mm. carapace width which had been collected in Lake Pontchartrain, Louisiana, by Drs. R. M. Darnell and R. D. Suttkus. From the evening of the day they were collected until the observations were completed the crabs were kept in aquaria partially filled with 25% sea water in an air-conditioned darkroom. Temperature was maintained at about 16° C. and the water in the aquaria was changed every

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second day. Sex was not taken into account in selecting the animals for study. The crabs were exposed to light only during the periods of observation which lasted only a few minutes each hour.

The melanophores of all the crabs were observed in the darkroom by the use of a stereoscopic dissecting microscope and microscope light at twelve hourly intervals beginning at 8 A.M. and the degree of dispersion of the pigment within the melanophores determined. The only exceptions occurred on March 5 and 6 when the chromatophores were observed hourly throughout the night. The chromatophore index of Hogben and Slome (1931) was employed to stage the melanophores. In this system the most concentrated condition of the melanin is described as stage 1, the most dispersed as stage 5, and the intermediate conditions as stages 2, 3, and 4. The melanophores of the anterior aspect of a walking leg of each crab were staged

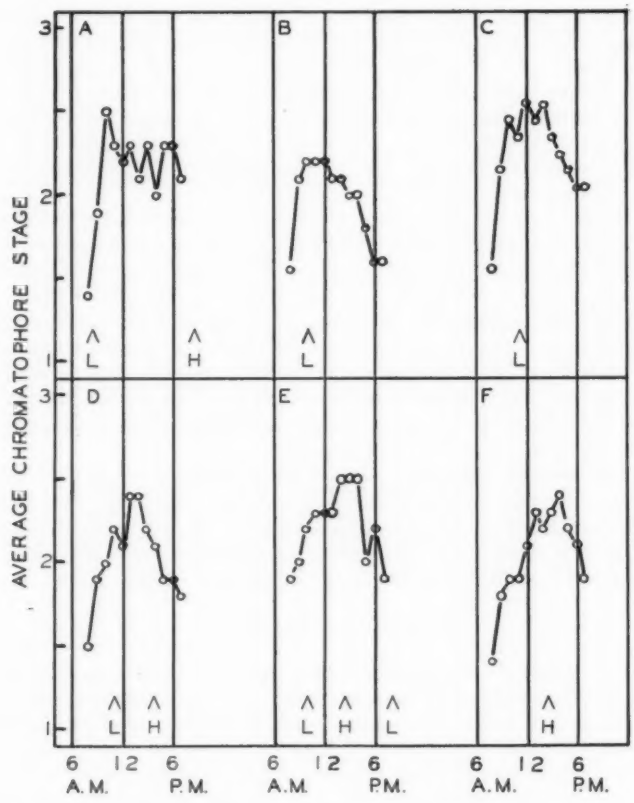

FIGURE 1. Changes in the black pigment of *Callinectes* in darkness. A is the record for Jan. 17, B for Feb. 19, C for Feb. 22, D for Feb. 24, E for Mar. 8, and F for Mar. 10.

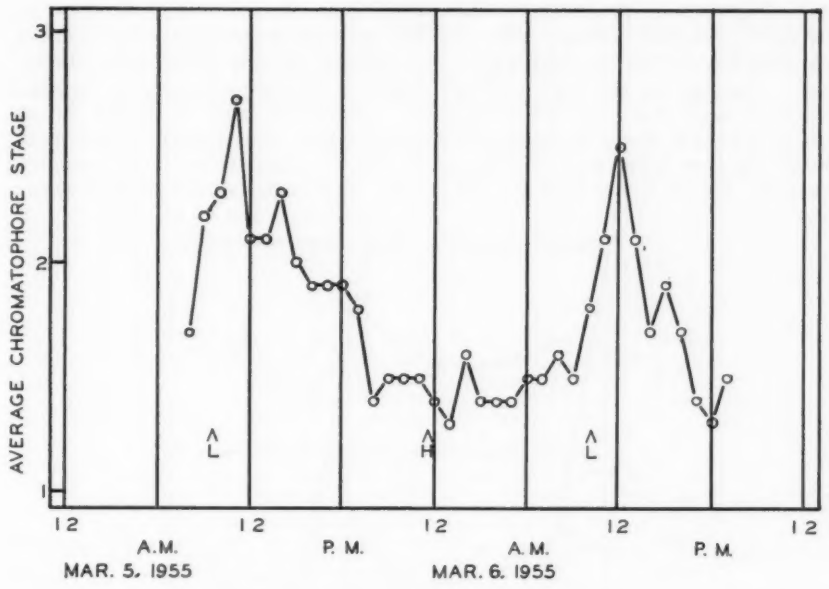

FIGURE 2. Changes in the degree of dispersion of the black pigment during a period in darkness.

and the average of the group was determined. The changes of coloration are not as striking in *Callinectes* as in *Uca*. In the former not only are there fewer melanophores per unit area on the legs but also the individual chromatophores of *Callinectes* do not exhibit the same high degree of branching that is found in *Uca* when the melanin is completely dispersed.

RESULTS AND DISCUSSION

A preliminary experiment (series A) performed on January 17th established the existence of a diurnal pigmentary rhythm (Fig. 1A). The crabs darkened by day and lightened by night. Twenty-nine animals were used.

A long period of observation (series B) occurred between February 19th and March 13th. These observations were begun with 52 animals. The number gradually diminished until only ten survived at the end of the observations. The hourly averages of the chromatophore stages for this series are presented in Table I. The second long period of observations (series C) was begun on March 5th and continued until March 13th. Twenty-one of the 28 *Callinectes* survived. The decrease in the number of animals had no effect upon the results. The reproducibility of the data obtained from the two long periods of observation (Fig. 3) indicates this. The hourly averages of the chromatophore stages for series C are presented in Table II. The times of low tide and high tide in Lake Pontchartrain on days observations of the chromatophores were made are presented in Table III.

In Figure 2 are presented the data obtained on March 5th and 6th from the *Callinectes* collected February 18th. In this figure the arrows below the curve indicate the times of low and high tide for these days in the original habitat of the animals. The pigment exhibited a diurnal dispersion and concentration; its maximal degree of dispersion occurred about noon. The major peak on March 5th occurred at 11 A.M. and a secondary peak was at 2 P.M. On March 6th both peaks shifted one hour to the right with the result that the principal maximum was at 12 noon and the secondary peak was at 3 P.M. This indicated that a tidal compo-

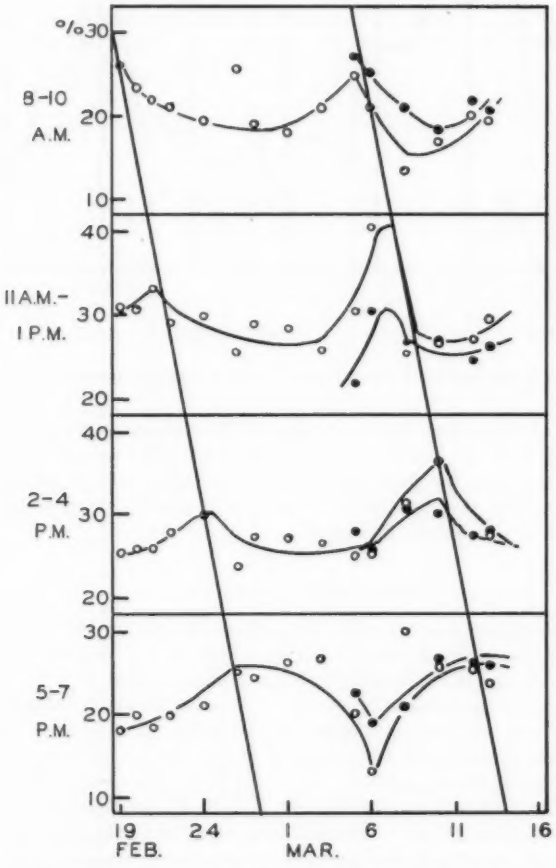

FIGURE 3. Relationship between the percentage of the daily melanin dispersion occurring at each of four periods during the day and the day of the month. Note the tidal maxima passing across the daily periods at the tidal rate of approximately fifty minutes per day. There is also a 14.5-day cycle, the interval between the diagonal lines. The circles represent animals collected Feb. 18, dots represent animals collected March 4.

TABLE I

The average melanophore index for each of the 12 daily measurements made during the first long period of observation (Series B)

Time	Date															
	February							March								
	19	20	21	22	24	26	27	1	3	5	6	8	10	12	13	
8 A.M.	1.6	2.2	1.5	1.5	1.5	1.7	1.3	1.3	1.6	1.7	1.7	1.4	1.4	1.8	1.7	
9	2.1	2.8	2.2	2.1	1.9	2.1	1.9	1.4	1.9	2.2	2.2	1.6	1.8	2.2	2.0	
10	2.2	2.8	2.3	2.4	2.0	2.0	1.9	1.7	1.7	2.3	2.3	1.6	1.9	2.0	2.0	
11	2.2	3.0	2.5	2.3	2.2	1.8	2.1	1.4	1.9	2.7	2.7	1.7	1.9	2.2	2.1	
12 M.	2.2	3.3	2.5	2.5	2.1	2.0	2.1	1.7	1.9	2.1	2.1	2.0	2.1	2.4	2.4	
1 P.M.	2.1	3.0	2.5	2.4	2.4	2.0	2.0	1.7	1.9	2.1	2.1	2.3	2.3	2.4	2.6	
2	2.1	2.9	2.3	2.5	2.4	1.8	2.2	2.0	1.9	2.3	2.3	2.3	2.2	2.8	2.6	
3	2.0	2.0	2.3	2.3	2.2	1.9	2.0	1.9	1.9	2.0	2.0	2.1	2.3	2.0	2.1	
4	2.0	2.5	1.9	2.2	2.0	1.9	1.8	1.7	2.0	1.9	1.9	2.3	2.4	2.3	2.3	
5	1.8	2.4	1.9	2.1	1.9	2.0	2.0	1.7	2.1	1.9	1.9	2.4	2.2	2.5	2.3	
6	1.6	2.5	1.9	2.0	1.9	2.0	1.9	1.7	1.9	1.9	1.9	2.2	2.1	2.1	1.9	
7	1.6	2.2	1.7	2.0	1.8	1.7	1.8	1.7	1.8	1.8	1.8	1.9	1.9	2.2	2.1	

nent was superimposed upon the diurnal rhythm and was progressing across the scale at the rate of about one hour per day.

In Figure 1 are shown the data obtained on six other days with the times of low and high tide in Lake Pontchartrain indicated by the arrows. These days were selected to illustrate best the observed types of modification of the diurnal rhythm curve when the low or high tide was (a) early in the morning, (b) moving across noon, and (c) in the early evening.

TABLE II

The average melanophore index for each of the 12 daily measurements made during the second long period of observation (Series C)

Time	Date					
	March					
	5	6	8	10	12	13
8 A.M.	1.9	1.6	1.9	1.4	1.7	1.7
9	2.0	1.5	2.0	1.6	2.1	2.1
10	2.0	1.8	2.2	1.9	2.3	2.0
11	1.8	2.1	2.3	1.9	2.4	2.0
12 M.	1.8	2.5	2.3	1.9	2.1	2.3
1 P.M.	1.7	2.1	2.3	1.9	2.0	2.3
2	1.8	1.7	2.5	2.1	2.3	2.3
3	1.7	1.9	2.5	2.0	2.6	2.3
4	2.5	1.7	2.5	2.1	2.0	2.2
5	1.8	1.4	2.0	2.2	2.1	2.3
6	1.8	1.3	2.2	1.9	2.5	2.0
7	1.8	1.5	1.9	1.6	2.1	2.0

TABLE III

The times of low and high tides during the days observations were made. The times are corrected for Lake Pontchartrain where the Callinectes were collected

Date	Low tide	High tide
Jan. 17	8:14 A.M.	8:38 P.M.
Feb. 19	9:56 A.M.	11:38 P.M.
20	10:27 A.M.	—
21	10:50 A.M.	12:33 A.M.
22	11:04 A.M.	1:26 A.M.
24	10:48 A.M.	3:35 A.M.
	10:44 P.M.	3.40 P.M.
26	12:57 A.M.	4:14 P.M.
27	3:00 A.M.	4:58 P.M.
Mar. 1	5:53 A.M.	6.51 P.M.
3	9:18 A.M.	10:35 P.M.
5	9:35 A.M.	11:43 P.M.
6	10:05 A.M.	—
8	10:07 A.M.	2:37 A.M.
	8:10 P.M.	2:29 P.M.
10	11:26 P.M.	2:22 P.M.
12	12.50 A.M.	2 46 P.M.
13	2.18 A.M.	3.38 P.M.

On January 17th (Fig. 1A) low tide was at 8:14 A.M. and led to a maximal response at 10 A.M. High tide was at 8:38 P.M., so late in the day that it kept the chromatophore pigments semidispersed throughout the day and into the evening. There was also the suggestion of a minimum at about 4 P.M., tending to produce a bimodal curve. This bimodality is due to the absence of a tide near the middle of the day. Therefore, the diurnal rhythm alone was functioning to maintain a peak near noon. On February 19th (Fig. 1B) as the low tide moved to 9:56 A.M. the peak shifted and the maximum degree of pigment dispersion occurred at approximately 11 A.M. The high tide progressed until it occurred so late in the evening that it had no effect upon the diurnal rhythm. Therefore, the pigment gradually concentrated throughout the afternoon because there was no evening tide to keep it dispersed. On February 22nd and 24th (Fig. 1, C and D) the time of the tide had advanced approximately one to two hours and these curves are skewed to the right of noon. The data in Figure 2 for March 5th and 6th would fit between Figures 1B and 1C if they had been plotted on the same graph. On March 8th (Fig. 1E) the major tide was at 2:29 P.M. and the curve has its maximum at approximately 3 P.M. This curve is, therefore, a mirror image of Figure 1B; the pigment here gradually dispersed throughout most of the day rather than gradually becoming concentrated as in Figure 1B. On March 10th (Fig. 1F) the curve is skewed even more to the right. The maximum occurred at 4 P.M.

Evidently, as the time of the tide progressively becomes later in the day, a tidal component moves across the diurnal rhythm at the same rate that the tides are moving. In general, the tide exerts its maximal effect one to two hours after the time of the high or low tide.

The tides of the Gulf of Mexico and its adjacent waters are generally diurnal, there being one low and one high tide every lunar day. But, for approximately three days of every tidal cycle the tides tend to become semidiurnal; there are two low and two high tides per lunar day. This phenomenon occurs twice each lunar

month when the moon is in the plane of the equator (Fig. 1, D and E). The data depicted in Figure 1D were obtained from observations of animals that were responding only to the time of low tide. It is obvious from the figure they continued to respond only to the time of low tide in spite of the appearance of a high tide later in the day. Furthermore, the response of the melanophores to a tide which occurred at that time of day did not lead to a curve with the shape of the one represented on February 24th (Fig. 1D). Three tides occurred during the hours of observation on March 8th (Fig. 1E), but the crabs responded only to the tide which occurred about 50 minutes later than on the previous day. A prediction based on the data of March 5th and 6th (Fig. 2) indicated that the peak which occurred at approximately 2 P.M. on March 8th (Fig. 1E) was to be expected. The two low tides which occurred at 10:07 A.M. and 8:10 P.M. could not have led to a curve with the shape of the one obtained on March 8th (Fig. 1E) because of the times of day at which they occurred.

The rhythmical tidal center in the animal is operating solely on the basis of tides that are spaced 12.4 hours apart, independent of whether they are both the same type of tide; and the shift to a semidiurnal tidal picture has no effect upon the center of tidal rhythmicity. This fact provides further evidence that these rhythms of *Callinectes* are endogenous and not directly influenced by the tides because the existence of a supernumerary tide has no influence upon the animals.

The *Uca* at Woods Hole, Massachusetts also show a response to tides spaced 12.4 hours apart (Brown, Fingerman, Sandeen and Webb, 1953). But the *Uca*, which were living in a permanently semidiurnal tidal situation, showed augmentation of the degree of dispersion of the melanin one to three hours after a low tide and not after a high tide. However, *Callinectes* exhibits a response not only to a low tide but also to a high tide which occurs for most of the tidal cycle 12.4 hours on the average after the low tide, which is the same interval of time that separates the two low tides at Woods Hole, Massachusetts.

To demonstrate the rate at which the tidal rhythm progressed over the diurnal rhythm, the following method of analysis was employed. The twelve periods of observation were divided into four periods of three hours each, 8–10 A.M., 11 A.M.–1 P.M., 2–4 P.M. and 5–7 P.M. The percentage of the total daily excursion that each three-hour period occupied was then calculated. This was done by summing the twelve average stages of the chromatophores which were obtained throughout the day and subtracting twelve from the total. If there had been no daily dispersion of the pigment, the sum of the indices would have been twelve. In like manner, the averages of the chromatophore indices for each three-hour period throughout the day were summed and three was subtracted from the daily total. The percentage of the total that each three-hour period occupied was then calculated. The data obtained in this fashion from series B and C have been used in the preparation of Figure 3.

The tidal maxima and minima travel across the scale at a rate closely approximating that at which a tidal cycle would be expected to progress (Fig. 3). This is illustrated by the two parallel diagonal lines drawn through the maxima at a distance of 14.5 days from one another. This is approximately the expected interval between days on which a low and a high tide will occur at the same time of day at any given location. Here again it is obvious that the animals are responding to both high and low tides; if they were responding to only one or the other, the maxima would be displaced from one another by approximately 29.5 days, which is

the interval between days on which a low tide or a high tide occurs at the same time of day at any given location where the tides are diurnal.

The similarity of the results obtained with both groups of animals is striking (Fig. 3), the maxima and minima occurring at approximately the same day of the month in both groups. Or in other words, the animals which were in the laboratory darkroom for fourteen days prior to the collection of the next group of animals continued to maintain their tidal and diurnal rhythms in synchrony with those animals which were still in the field subject to the rhythmic variations of tide and light.

In Table IV are listed the daily amplitudes of the diurnal rhythm for series B and C. These values are the total of the average melanophore stages for the twelve daily measurements. There is no apparent increase in the amplitude of the rhythm with time in darkness. Brown, Fingerman, Sandeen and Webb (1953) showed that the amplitude of the diurnal rhythm of *Uca* gradually increased day by day until a maximum was reached in about ten days. The value for the second

TABLE IV

The amplitude of the diurnal rhythm of animals maintained in darkness. The amplitude is expressed as the total of the average stages for the 12 daily measurements of the melanophores

Date	Series B	Series C
Feb. 19	23.2	
20	32.5	
21	25.5	
22	26.3	
24	24.4	
26	22.9	
27	23.0	
Mar. 1	21.5	
3	22.5	
5	24.9	22.6
6	21.1	25.8
8	24.1	26.6
10	24.5	22.4
12	26.9	26.2
13	25.9	25.7

day of series B is much higher than ordinarily observed which probably is due to the fact that on this one day the air-conditioner failed and the darkroom was much warmer than on any other day. This indicates that the amplitudes of the rhythms are dependent upon temperature although their frequencies are independent of temperature.

GENERAL DISCUSSION

Melanophore pigment rhythms of *Callinectes sapidus* have precise frequencies and must, therefore, be temperature-independent within the normal physiological range. If they were not temperature-independent they would have no adaptive significance to poikilothermic or heterothermic animals.

In one respect investigation of the pigmentary rhythms of *Callinectes* is more advantageous than with *Uca* because the amplitude of the diurnal pigment migration of *Callinectes* does not increase with time in darkness as it does in *Uca*. As a result, all phases of the tidal and diurnal rhythms exhibit a maximal expression

throughout the entire period of the observations and are not obscured after a period of about ten days as they are in *Uca*.

The 12.4-hour frequency of the tidal rhythm in blue crabs is interesting because of the diurnal nature of the tides in its natural habitat. *Callinectes* responds in the same manner to high and low tides when these tides occur at the same time of day, whereas *Uca* responds only to low tides in the semidiurnal tidal habitat near Woods Hole, Massachusetts.

The evolution of tidal rhythms may have resulted from the existence of two events 12.4 hours apart to which the animals were exposed. The tidal rhythm may now be independent of the original events by virtue of an endogenous timing mechanism. In the evolution of precisely timed biological rhythms the nature of the two events which occurred at a set frequency may have been of secondary importance to the set frequency itself which in time became impressed upon the organism as a deep-seated phenomenon.

The interaction of the diurnal and tidal pigmentary rhythms in *Callinectes sapidus* is a basis for long term rhythms, just as found in *Uca*, because the tidal and diurnal rhythms come into synchrony only once every 14.8 days approximately, which is half a lunar cycle. If the *Callinectes* had been responding differently at times correlated with high and low tides, they would have been in an identical condition physiologically only once every lunar cycle.

The continued response of *Callinectes* to a diurnal tidal situation on days that the tides in its habitat become semidiurnal demonstrates that the tidal rhythm is a deep-seated phenomenon and is not easily altered by environmental conditions.

Summary

1. The pigment in the melanophores of *Callinectes sapidus* displays an endogenous diurnal rhythm with a frequency of 24.0 hours. The pigment is in the dispersed state during the day and in the concentrated state at night.

2. The amplitude of the diurnal rhythm neither increases nor decreases under constant laboratory conditions.

3. Superimposed upon the diurnal rhythm is a tidal rhythm with a frequency of 12.4 hours. This rhythm is manifested by a supplementary dispersion of the melanin which occurs about fifty minutes later each day.

4. The tidal rhythm is most evident when the low or high tide is either in the morning or late afternoon. When either a low tide or a high tide occurs at these times, the diurnal rhythm curve is skewed to the left or right or tends to be bimodal, depending upon the times of high and low tides. There is no difference of response to low and high tides.

5. The tidal rhythm of *Callinectes* maintained under constant laboratory conditions has its phases bearing a definite relationship to the times of low tide and high tide in the native habitat of the crabs.

6. The *Callinectes* continue to exhibit their response to tides spaced 12.4 hours apart even on days that the usually diurnal tidal cycle of their original habitat becomes semidiurnal.

7. *Callinectes* also exhibits a semilunar rhythm. Only once every 14.8 days are the diurnal and tidal rhythms in the same phases relative to one another.

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MORPHOGENETIC MOVEMENTS OF NORMAL AND GASTRULA-ARRESTED HYBRID AMPHIBIAN TISSUES

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From a certain morphological point of view, some hybrid embryos behave like normal embryos treated appropriately with chemical inhibitors: they begin to gastrulate and then stop. Embryos belonging to the hybrid *Rana pipiens* ♀ × *Rana sylvatica* ♂, for example, stop gastrulating almost as soon as they begin (Moore, 1946). Externally, however, they look very much like ordinary *Rana pipiens* embryos that have been arrested at the same stage by exposure to 2,4-dinitrophenol or other metabolic poisons (Ornstein and Gregg, 1952). In fact, direct observation of blocked embryos generally does not yield evidence relevant to classifying genetical or chemical inhibitors according to their effects upon morphogenetic movements of different sorts—although the parts of normally gastrulating embryos severally undergo complexly interrelated displacements of different morphological kinds (see Nelsen's classification, 1953). Recently, however, Holtfreter (1944) has discovered that many normally-occurring gastrular movements can be modelled in isolation by movements that take place in suitably constructed explant systems; and application of his techniques to chemically treated tissues has already provided some warrant for the belief that gastrular movements are differentially retarded or suppressed by some metabolic poisons (Gregg and Ornstein, 1953). In the work about to be reported, we have extended Holtfreter's methods to study the capacities of gastrula-arrested hybrid tissues to undergo surrogate morphogenetic movements. On the basis of the results, we shall try to explain in morphological terms why hybrids of the kind studied fail to gastrulate.

Methods

General

Appropriate explants were obtained by dissecting *Rana pipiens*, *Rana sylvatica* and *Rana pipiens* ♀ × *Rana sylvatica* ♂ Stage 10 (Shumway, 1940) embryos in Holtfreter's solution (often without bicarbonate, usually with 0.1 gm. sodium sulfadiazine per liter, at pH's ranging from 5.5 to 8.4). These were assembled into explant systems whose behavior was observed intermittently for periods up to 48 hours, at temperatures ranging from 9° to 25° C.

For our purposes, we shall say that the environmental conditions indicated constitute the set of *standard* conditions, since the morphological characteristics of the tissue movements that we have studied do not seem to be functionally related to environmental changes of the specified sorts.

For an account of explant systems more detailed than that given here, the reader is referred to the paper by Gregg and Ornstein (and also to that of Holtfreter). However, the systems discussed therein differ from some of those of

present concern, and some brief descriptive and interpretative remarks are thus in order.

Type I. (Systems of this type not used in investigations now being reported.)

Type II. Exactly as described by Gregg and Ornstein. Rider consists of some presumptive notochord set uncoated side down on uncoated surface of yolk endoderm base. Endodermal part of rider expected to embed in base in movement (called "endodermal embedding in endoderm," or, briefly, "Em(en,en)") construed as homolog of bottle-cell formation. Mesodermal part of rider expected to elongate in its original meridional axis in movement (called "mesodermal stretching on endoderm," or, briefly, "St(m,en)") construed as homolog of convergent stretching.

Type III. (Systems of this type not used in investigations now being reported.)

Type IIIa. Systems of this kind like those of type III, except for omission of mesodermal component. Surface-coated ectodermal rider set uncoated side down on uncoated surface of yolk endoderm base. Rider expected to spread in sheet over base in movement (called "ectodermal spreading on endoderm," or, briefly, "Sp(ec,en)") construed as homolog of epibolic spreading.

Type IV. System consists solely of explanted Type II-system rider. Behavior expected: explant heals, becoming spherical; mesodermal portion elongates in movement (also called "mesodermal stretching on endoderm," or, briefly, "St′(m,en)"—the accent marks the difference, if any, from St(m,en)) construed as homolog of convergent stretching.

Presentation of data

We shall present our work in the manner of Gregg and Ornstein: the data will be summarized in a sequence of abbreviated expressions, interspersed with commentary. Because the systems dealt with here are of more complex origin than theirs, we shall have to complicate their abbreviatory conventions in detail, though not in principle.

The general abbreviatory form is:

$$M(g_t,g'_{t'})S{:}C;\ (k)n\mathrm{P};\ q\mathrm{N};\ r\mathrm{I}$$

where:

M is the sort of movement studied: stretching (St,St′), embedding (Em), spreading (Sp);

g and g' are the germ-layer types of the riders and the bases, respectively: endoderm (en), mesoderm (m), ectoderm (ec), (for Type IV systems, the endodermal portion will be regarded as base, the ectodermal portion as rider);

t and t' are the taxonomic types of the gastrulae supplying the riders and bases, respectively: *pipiens* (p), *sylvatica* (s), hybrid (h);

S is the type of explant system: II, IIIa, IV;

C is the set of environmental conditions: standard in every case;

k, n, q, and r are natural numbers; and

P is the set of positive results, N is the set of negative results, and I is the set of indeterminate results.

Thus, for example:

$$St(m_s,en_p)II: \text{standard}; (8); 7P; 1N; 0I$$

is read as asserting that of our attempts to obtain stretching of *sylvatica* mesoderm on *pipiens* endoderm, using Type II systems under standard conditions, 7 out of 8 were successful, 1 out of 8 was unsuccessful, and none yielded indeterminate results.

Slight departures from these conventions (see (23)–(28)) will be explained when the need arises.

Results

Morphogenetic movements of normal tissues

(2)–(4) merely confirm the results of Gregg and Ornstein in showing that *pipiens* tissues undergo the expected movements in explant systems:

(1) $Em(en_p,en_p)II$: standard; (7); 6P; 1N; 0I

(2) $Sp(ec_p,en_p)IIIa$: standard; (76); 67P; 6N; 3I

(3) $St(m_p,en_p)II$: standard; (22) 17P; 5N; 0I

(4) $St'(m_p,en_p)IV$: standard; (44); 38P; 1N; 5I

(5) shows that the tissues of *sylvatica* gastrulae, also, can exhibit some of these responses:

(5) $Sp(ec_s,en_s)IIIa$: standard; (4); 3P; 1N; 0I

Unfortunately, we do not have any direct information about $Em(en_s,en_s)$, $St(m_s,en_s)$ or $St'(m_s,en_s)$, although results with interspecific tissue combinations ((7)–(12), below) strongly suggest that these movements can be made to occur unorthodoxly, and that *sylvatica* tissues do not differ from *pipiens* tissues in their ability to exhibit these movements (note (10), however).

Explant systems whose components derive from embryos belonging to different species behave, nevertheless, in generally normal fashion:

(7) $Em(en_p,en_s)II$: standard; (4); 4P; 0N; 0I

(8) $Em(en_s,en_p)II$: standard; (8); 7P; 1N; 0I

(9) $Sp(en_p,en_s)IIIa$: standard; (4); 4P; 0N; 0I

(10) $Sp(ec_s,en_p)IIIa$: standard; (10); 4P; 4N; 2I

(11) $St(m_p,en_s)II$: standard; (5); 4P; 1N; 0I

(12) $St(m_s,en_p)II$: standard; (8); 7P; 1N; 0I

(We do not have information about St'(m,en) in Type IV systems with interspecific tissue combinations.) In general, (7)–(12) are confirmatory of Holtfreter's claim that embryonic tissue affinities are not species-specific; the exception, (10) suggests that *sylvatica* ectoderm does not spread well on *pipiens* endoderm but further observations will be required to settle this point definitely (see (22)).

Morphogenetic movements of hybrid tissues

Embryos belonging to the hybrid *R. pipiens* ♀ × *R. sylvatica* ♂ will begin to gastrulate—each embryo finally exhibits a dorsal lip and develops an abbreviated archenteron. Therefore, if movements of the sort Em(en,en) are homologous with certain of those which accompany dorsal lip inauguration (*i.e.*, bottle-cell formation in the gray crescent area), then one would expect hybrid tissues to exhibit Em(en,en). This expectation is well-founded:

(13) $Em(en_h,en_h)II$: standard; (16); 16P; 0N; 0I

As a matter of fact, hybrid presumptive head endoderm embeds also in normal yolk endoderm:

(14) $Em(en_h,en_p)II$: standard; (9); 9P; 0N; 0I

(15) $Em(en_h,en_s)II$: standard; (5); 5P; 0N; 0I

and hybrid yolk endoderm provides a substratum in which normal presumptive head endoderm can embed:

(16) $Em(en_p,en_h)II$: standard; (4); 4P; 0N; 0I

(17) $Em(en_s,en_h)II$: standard; (8); 8P; 0N; 0I

Thus, insofar as Em(en,en) is concerned, hybrid tissues and normal tissues seem to behave alike.

But hybrid embryos only rarely gastrulate past Stage 10, and it is reasonable, therefore, to expect hybrid tissues to fail in respect to some of the other gastrular movements that it is possible to model in explant systems.

The evidence with respect to movements of the kind Sp(ec,en) is somewhat complex. Hybrid ventral ectoderm spreads well on hybrid yolk endoderm:

(18) $Sp(ec_h,en_h)IIIa$: standard; (13); 11P; 0N; 2I

and also on normal yolk endoderm:

(19) $Sp(ec_h,en_p)IIIa$: standard; (8); 7P; 1N; 0I

(20) $Sp(ec_h,en_s)IIIa$: standard; (3); 3P; 0N; 0I

But hybrid yolk endoderm appears to be less well suited than normal yolk endoderm as a substratum upon which normal ventral ectoderm can spread:

(21) $Sp(ec_p,en_h)IIIa$: standard; (20); 12P; 8N; 0I

(22) $Sp(ec_s,en_h)IIIa$: standard; (8); 4P; 3N; 1I

Before accepting this interpretation of (21) and (22)—that the high frequency of negative results is indicative of some peculiarity of hybrid yolk endoderm in respect to movements of the sort Sp(ec,en)—the reader should recall that *sylvatica* ectoderm seems to spread with a relatively low frequency of success upon normal yolk endoderm, as well (10).

With respect to models of convergent elongation the situation is more clear-cut, for explanted hybrid presumptive notochord invariably fails to elongate, no matter

what the endodermal substratum provided:

(23) St(m_h,en_h)II: standard; (15); 0P; 15N(spread); 0I

(24) St'(m_h,en_h)IV: standard; (26); 0P; 26N(spread); 0I

(25) St(m_h,en_p)II: standard; (9); 0P; 9N(spread); 0I

(26) St(m_h,en_s)II: standard; (5); 0P; 5N(spread); 0I

What does happen in experiments of the sorts whose results are reported in (23)–(26) is that *hybrid presumptive notochord behaves toward an endodermal substratum like ventral ectoderm and spreads over it* instead of elongating in the fashion typical of presumptive notochord from normal embryos. (This is the significance of the word "spread" inserted after the itemizations of negative results in (23)–(26).)

(25) and (26) suggest that, for the failure of Type II and Type IV systems to exhibit movements of the sorts St(m_h,en) and St'(m_h,en), blame will have to be shouldered by hybrid presumptive notochord, which fails to elongate even upon normal endodermal substrata. On the other hand, there is at least some evidence that hybrid yolk endoderm is not completely satisfactory as a substratum for normal mesodermal activities of this kind:

(27) St(m_p,en_h)II: standard; (4); 2P; 1N(spread); 1I

(28) St(m_s,en_h)II: standard; (8); 4P; 2N(spread); 2I

In fact, (27) and (28) suggest that the presence of hybrid endoderm as a substratum can even stimulate tissues to spread—*i.e.*, *pipiens* and *sylvatica* presumptive notochord—that behave in quite a different manner when explanted upon normal yolk endoderm. But we view this interpretation of (27) and (28) with some misgivings. If one attempts to explant presumptive notochord from gastrulae that are well into Stage 10, it is always possible that one mistakenly obtains ectoderm (presumptive neural plate) instead of mesoderm because of the difficulty of locating presumptive areas in partly gastrulated embryos—especially in the dorsal lip region. Whenever possible, we have dissected gastrulae as soon as their dorsal lips were detectable, but it is at least conceivable—we cannot decide more definitely about this point—that some tissues that we thought were mesodermal were actually ectodermal and that the seemingly negative results in (27)–(28) are explainable on this basis. Further evidence is needed to settle this question.

Discussion

We believe that our results provide us with certain key data for a morphological explanation of the fact that hybrid embryos begin to gastrulate and then stop.

Our proposal is this. When embryos gastrulate normally, their presumptive notochords invaginate; and, whenever presumptive notochords invaginate, their cells converge medially (an unambiguous diagram is given in Barth's *Embryology*, p. 121, Fig. 6.14). Now, from Moore's description of hybrid embryos, it is clear that their head endoderms invaginate and come to underlie their presumptive notochords. We have shown ((23)–(26)) that whenever hybrid presumptive noto-

chords are provided with endodermal substrata in explant systems, they spread like ectoderm instead of converging medially like normal presumptive notochords; and we assume that something similar happens in intact hybrid embryos—that after invagination of their head endoderms has provided their presumptive notochords with endodermal substrata, the tendency of their notochordal mesoderms to spread in all directions upon these substrata effectively overcomes whatever tendencies their mesoderms have to converge medially. It follows that hybrid embryos cannot gastrulate normally.

Of course, it may be the case that hybrid notochordal mesoderms have *no* tendencies to converge medially: this in itself might be a sufficient reason why hybrid embryos do not gastrulate normally. To obtain information on this point, we explanted, without head endoderm, 9 hybrid presumptive notochords, all of which remained roughly spherical after healing, and 7 *pipiens* presumptive notochords, 5 of which thereupon exhibited typical notochordal elongation. Thus, our first explanation may explicate only one of several morphological reasons why hybrid embryos fail to execute normal gastrular movements. In any case, hybrid presumptive notochords are decidedly abnormal in their capacities to perform morphogenetic movements.

Summary

1. Explant systems have been constructed to test the abilities of isolated normal (*Rana pipiens*, *Rana sylvatica*) and hybrid (*R. pipiens* ♀ × *R. sylvatica* ♂) gastrular tissues to undergo activities surrogative of certain morphogenetic movements occurring in gastrulating embryos.

2. Evidence has been obtained to show that hybrid ventral ectoderm can carry out normal morphogenetic movements. On the other hand, there is some indication (see (10), (21), (22), (27) and (28)) that hybrid yolk endoderm is abnormal; and there is definite evidence ((23)–(26)) to show that the behavior of hybrid presumptive notochord is deviant from that of normal presumptive notochord.

3. Some tentative explanations are offered in morphological terms for the failure of hybrid embryos to gastrulate past Stage 10.

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CHANGES IN THE PROTOPLASM DURING MATURATION[1]

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In recent years there has been a great interest in the physiology of cell division, and in many laboratories in various parts of the world investigators have attempted in so far as possible to obtain data that would help to elucidate the series of events that occur when a cell divides. From the standpoint of a physiologist, one of the most essential problems is the question as to the nature of the stimulus that induces a cell to begin the process of division. In order to find an answer to this question, a physiological attack is necessary; for no amount of morphological information, or morphological information combined with chemical recognition of morphological entities, can presumably offer much help. Certainly it has not been of much help thus far. What is needed is information as to which agents will cause cells to divide, which agents will prevent them from dividing, and why these agents act as they do. For this type of study marine egg material is ideal.

In our earlier work, we have been able to show that all the agents that induce marine eggs to divide cause a clotting of the protoplasm, a clotting typically inspired by the release of calcium from the cortex of the cell. The released calcium activates an enzyme system which is at the same time both a protease and a clotting enzyme. For a discussion of this earlier work, see Heilbrunn (1952a, 1952b), Heilbrunn, Chaet, Dunn and Wilson (1954), Heilbrunn and Wilson (1955), and Heilbrunn (1955). It is doubtless true that anything that causes a sea urchin egg or an egg of the worm Chaetopterus to undergo the first cleavage division, first causes a protoplasmic gelation, the so-called mitotic gelation. This is true both for the natural stimulus of fertilization and also for artificial stimuli of one sort or another.

But let us consider the case of immature eggs. These, too, following a proper stimulus (or the release of an inhibition), begin a division process. This process may involve only the splitting off from the large egg of a tiny cell, but the process is a division process nevertheless. And in an immature egg, with a large nucleus or germinal vesicle, the stimulus to divide is followed by a liquefaction of the protoplasm. How can this apparent contradiction be explained on the basis of our theory?

This was the problem we set for ourselves. It might be thought that anything which caused the breakdown of the germinal vesicle would release some substance which would liquefy the protoplasm. But there is also another possibility. If the calcium that is released as a result of stimulation does indeed activate an enzyme which is both a clotting enzyme and a protease, then such an enzyme might cause either a clotting of the protoplasm or a liquefaction—or it might indeed cause both.

[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, Public Health Service.

This idea appealed to us. In order to test it, we decided to determine whether the liquefaction of the protoplasm, which occurs when the maturation process begins, precedes or follows the breakdown of the germinal vesicle. If the liquefaction precedes germinal vesicle breakdown, then it could scarcely be held that the liquefaction was due to some substance released into the cytoplasm from the germinal vesicle nucleus. Also there might be a possibility that prior to the liquefaction there might be a mitotic gelation; although to tell the truth, when we began our investigation, we had small hope of obtaining such a result.

The viscosity of the protoplasm of the immature Chaetopterus eggs was determined by centrifuging pieces of egg-containing parapodia in a hand centrifuge of the type used in earlier studies from our laboratory. In order to make the tests rapidly, the handle of the centrifuge was turned once every second. This gave a force approximately 9,000 times gravity. Then at various time intervals after the eggs had entered sea water, the viscosity was measured in the same way. At first the results were not very uniform because of the fact that when the eggs were cut into sea water, some of the eggs came into contact with the sea water before others. Accordingly, it was found advisable to cut the worms on a piece of cheese-cloth, and then stir up the eggs that had emerged on to the cheese-cloth in sea water.

Figure 1 shows the results obtained at 21° C. The viscosity values are given as four times the number of seconds required to show zoning of the eggs. The reason why the number of seconds was multiplied by four is that earlier studies of the viscosity of the protoplasm of Chaetopterus eggs were made by turning the handle of the centrifuge once every two seconds. This gives a centrifugal force one-fourth that developed when the handle of the centrifuge is turned once every second. It might be thought that the protoplasm of immature eggs was thixotropic and that therefore values for the viscosity would vary with the shearing force. However, a series of tests at different centrifugal speeds, that is to say, at different shearing forces, showed that if the protoplasm is thixotropic all, the thixotropy is slight, and that viscosity values obtained at different shearing forces are approximately the same.

From Figure 1 it is clear that the viscosity of the immature egg doubles within a half minute after the egg enters sea water. Following this, the viscosity drops rapidly, so that by the time the egg has been in sea water for just one minute, the viscosity has returned to the value it had when the egg was in the parapodium. By the fifth or sixth minute after entrance of the egg into sea water, the viscosity of the egg protoplasm has dropped so low that only three seconds of turning the centrifuge at one turn of the handle per second are necessary to produce sufficient movement of the granules to show zones. This represents a value for the viscosity of about 12, although, of course, in view of the fact that the number of seconds of turning required was so small at this point, no great accuracy was possible. Thus if anything more than two seconds were required to shift the granules, the value was recorded as three. In earlier work (Heilbrunn and Wilson, 1948), the viscosity of the unfertilized egg after maturation was completed was found to be about 11, so that we can assume that after the fifth or sixth minute of immersion in sea water, any further decrease in the viscosity of the protoplasm is slight. At 21° C., the germinal vesicle breaks down at the seventh minute, so that at this temperature the liquefaction of the protoplasm certainly precedes the breakdown of the germinal vesicle.

Hence we can assume that the liquefaction of the protoplasm that occurs in the Chaetopterus egg is not due to some substance liberated from the nucleus, for it occurs before the nucleus shows any signs of breaking down. Our results thus indicate that the liquefying effect is due to the protease described by Goldstein (1953). According to Goldstein, as soon as the Chaetopterus egg enters sea water, a proteolytic enzyme system is activated by the entrance of calcium into the cell interior. Goldstein believes that this protease is responsible for the breakdown of

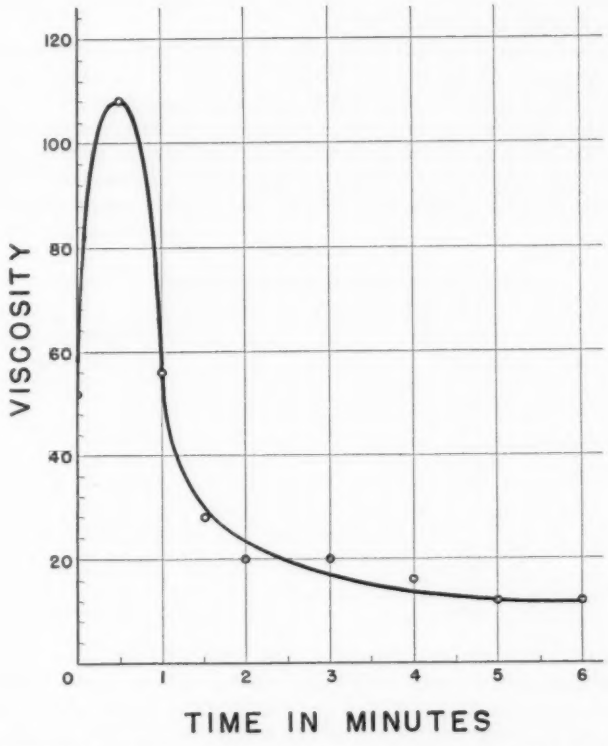

FIGURE 1. The viscosity of the protoplasm of the Chaetopterus egg at various intervals of time after the egg has entered sea water.

the germinal vesicle; our data seem to show that it also causes a liquefaction of the protoplasm. But before this liquefaction occurs, the protoplasm shows a very brief clotting reaction and this can be interpreted as constituting the mitotic gelation. We believe that both the gelation and the subsequent liquefaction can be caused by the same enzyme, for it is well known that calcium-activated proteases can act to produce both clotting and liquefaction. Indeed, thrombin itself is both a clotting and a proteolytic enzyme. For further discussion, see Heilbrunn (1955).

The curve shown in Figure 1 represents but a single set of data. It is supported by other data of the same sort obtained in other experiments at 21° C. Moreover, we did series of tests at various temperatures other than 21°. At temperatures from 6° to 24° C., it was always possible to show that the protoplasm became more fluid before the germinal vesicle broke down. At temperatures from 27° to 40° C., the germinal vesicle broke down very rapidly—in three to four minutes—and it was scarcely possible to observe any decrease in viscosity before the germinal vesicle broke down. At the lower temperatures, there seemed to be evidence of a rise in viscosity before the drop in viscosity occurred, but this was not followed as carefully as it was at 21° C.

Our primary interest was not in the effect of temperature on the rate of germinal vesicle breakdown. However, because the presence or absence of a germinal vesicle could rather easily be observed in the centrifuged eggs, it was possible for us to determine the time required for germinal vesicle breakdown at different temperatures. The data are not very remarkable, but they are presented here in the thought that they might be of interest to those who are concerned with the effect of temperature on various phases of mitosis. The results are given in Table I.

TABLE I

Time required for breakdown of the germinal vesicle of the Chaetopterus egg at various temperatures

Temperature	Time in minutes
6° C.	107
9	28
12	16
15	13
18	9
21	7
24	5
27	4
30	3
33	3
36	3
39	3
40	4

The question that we asked ourselves has certainly been answered. The initiation of maturation in the egg of Chaetopterus beyond any doubt causes the protoplasm of the egg to become more fluid even before the germinal vesicle breaks down. In preliminary experiments, we found this also to be true for the egg of the clam *Spisula solidissima*. In the Spisula egg, maturation does not begin until the egg is fertilized. Also, in the Chaetopterus egg, the liquefaction of the protoplasm is preceded by a transient increase in viscosity, indicative of a mitotic gelation. This mitotic gelation presumably is the precursor of the spindle of the first maturation division.

Our results indicate that when calcium is released into the protoplasm, it can cause a clotting reaction and it can also dissolve the clot or gel once it is formed. The double effect is due to the fact that calcium activates an enzyme which is both a clotting enzyme and an enzyme which has a proteolytic effect. There is an abundance of literature to indicate that such enzymes do exist. Indeed, clotting enzymes

such as rennin and thrombin are also proteolytic, and proteolytic enzymes such as trypsin can act as clotting agents. The subject is discussed at some length by Heilbrunn (1955) and references to the literature are given there. The facts as presented here serve to explain what has been regarded as a rather anomalous effect of calcium on the protoplasm of the squid giant axon. In a study of this protoplasm, Hodgkin and Katz (1949) found that calcium ion exerted a dispersing or liquefying action and their work has been widely quoted. As a matter of fact, in the Hodgkin and Katz experiment, the protoplasm was probably gelled as a result of injury (see Heilbrunn, 1955). If calcium activates a proteolytic enzyme (which is also a clotting enzyme), calcium could either cause clotting or liquefaction.

Summary

When an immature egg of the worm Chaetopterus enters sea water, the protoplasm first undergoes a transient increase in viscosity, and then rapidly becomes more fluid than it was originally. These effects are believed to be due to a calcium-activated proteolytic enzyme which is also a clotting enzyme. In the course of the investigation, data were obtained as to the effect of temperature on the rate of germinal vesicle breakdown.

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THE CONTROL OF NEUROSECRETION AND DIAPAUSE BY PHYSIOLOGICAL CHANGES IN THE BRAIN OF THE CECROPIA SILKWORM [1]

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Twenty-six neurons control the growth and metamorphosis of the Cecropia silkworm. These are the neurosecretory cells of the brain—nerve cells specialized for endocrine function. In each brain the neurosecretory cells are gathered into four distinct groups which in favorable preparations can be seen as clusters of bluish-white spots when the living brain is exposed and viewed *in situ* through a dissecting microscope (Williams, 1948, 1952). The hormone secreted by these cells directs a series of events which result in the insect's growth. There is now ample evidence to show that the brain hormone acts primarily on a single organ, the prothoracic glands. By this tropic action the brain elicits the secretion of the prothoracic gland hormone which then circulates in the blood and reacts with the tissues to favor growth and metamorphosis (Williams, 1947, 1954).

In circumstances where the brain fails to secrete its hormone, growth comes to a standstill. Such an eventuality, as Williams (1946b) first showed, normally occurs in insects with a pupal diapause. Here the failure of the neurosecretory cells to secrete the brain hormone after pupation is the basis of the pupal diapause. Months later the neurosecretory cells release the brain hormone and development is resumed.

The return of activity to the brain's neurosecretory cells is known to depend on the temperature history of the pupa (Williams, 1946b, unpublished data). In pupae stored at room temperatures a minimum of five or six months must elapse before the neurosecretory cells secrete the hormone and thereby terminate the pupal diapause. Yet, when similar pupae are given preliminary exposure for two or three months to temperatures which mimic those of the normal winter environment, a remarkable change occurs in the functional capacity of the neurosecretory mechanism. The brain, after the low temperature treatment, is now able to secrete its hormone promptly at the room temperature. Presumably, the low temperature acts directly on the brain and its neurosecretory cells, for, as Williams (1946b) demonstrated, the implantation of a chilled brain into an unchilled pupa causes the latter promptly to begin adult development.

How the low temperature restores "competency" to the neurosecretory cells is a mystery. Indeed, *Rhodnius prolixus* is the only insect where the mechanism controlling the activity of the brain's neurosecretory cells has been analyzed. Wigglesworth (1933, 1934, 1940) showed that the release of brain hormone is triggered

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by the enormous distension of the insect's abdomen following a blood meal. Since transection of the ventral nerve cord blocks this effect, it was inferred that the neurosecretory cells are driven by nerve impulses arising in the abdominal proprioceptors.

Furthermore, in experiments on vertebrates (summarized by Scharrer and Scharrer, 1954) there is increasing evidence that hormone release by the hypothalamic neurosecretory cells is triggered by nerve impulses—a conclusion strengthened by the recent study of Schimazu *et al.* (1954). Additional information concerning the control of neurosecretory cells is obviously desirable, especially in view of the widespread occurrence of neurosecretory cells in both vertebrates and invertebrates. The brain of the Cecropia silkworm appeared to be a favorable object for further analysis, since by varying the environmental temperature the endocrine activity of the neurosecretory system can be regulated at the convenience of the investigator. The observations of Wigglesworth encouraged the use of neurophysiological techniques for relating nervous and neurosecretory activities.

Materials and Methods

1. *Experimental animals*

Caterpillars of the giant American silkworm, *Platysamia cecropia,* were reared under nets on wild cherry trees. After pupation the cocoons were promptly harvested, stored temporarily at room temperature for about one month, and then subdivided into groups and placed at 6°, 15°, or 25° C. A smaller number of experiments were performed on the closely related saturniids, *Telea polyphemus* and *Samia walkeri* (*cynthia*). Several studies were performed on the wax moth, *Galleria mellonella,* a non-diapausing lepidopteran which was reared in the laboratory at 33° C. on a mixture of Pablum and glycerol.

During adult development the stage of development can be estimated by reference to the detailed morphological time table of Schneiderman and Williams (1954). In the absence of detectable morphological changes during the interval between the transfer of diapausing pupae from 6° C. and the onset of adult development at 25° C., it was necessary to judge the status of the animals on a statistical basis. A homogeneous group of 116 pupae was transferred to 25° C. after chilling at 6° C. for 13 weeks. Of these, 93 were used for physical or chemical measurements before the visible onset of adult development. The absence of development was confirmed in all cases by dissection. The remaining 23 pupae were used to determine the time for the initiation of adult development; the median time was 9 days, with extremes of 7 and 16 days.

2. *Methods*

a. *Electrical activity.* A Grass P-3 preamplifier and a Dumont 304-A oscillograph were used. The recording electrodes were No. 50 gauge platinum wires, or electrolytically tapered stainless steel insect pins (Grundfest *et al.*, 1950; Burkhardt, 1954), or indium-filled glass microelectrodes (Dowben and Rose, 1953). A Grass S-4 stimulator and stimulus isolation unit were used for electrical stimulation. The stimulation was through tapered silver-silver chloride electrodes (Roeder,

1946). All electrodes were manipulated by micromanipulators similar to that described by Goldacre (1954), but made from "Lucite" rather than glass.

The integument overlying the brain (or ganglia of the ventral cord) was removed and the active electrode inserted into the nervous tissue by use of the manipulator. The indifferent electrode consisted of a stainless steel needle inserted through the integument at the anterior end of the animal and extending into the hemolymph for about two centimeters.

b. *Cholinesterase.* Two different techniques were used for the assay of cholinesterase (ChE); namely, a colorimetric and a manometric technique.

In the colorimetric analysis 4 brains or 9 to 12 ganglia were excised from animals previously anesthetized with carbon dioxide (Williams, 1946a). The tissues were rinsed in insect Ringer (Ephrussi and Beadle, 1936) and ground in a small, conical, glass homogenizer. The resulting homogenate was diluted by the addition of 3.6 ml. of a solution containing 0.56 *M* NaCl and 0.063 *M* KH_2PO_4; the pH of this solution had previously been adjusted to 8.0 by the addition of NaOH (Chadwick *et al.*, 1953). The homogenates, after dilution, were stored in the deep freeze at − 20° C. One to 42 days later the assay was performed. The homogenate was equilibrated with 25° C. in a water bath, and then 0.4 ml. of 0.15 *M* acetylcholine bromide (ACh, Eastman "reagent grade") was added to yield a final volume of 1.0 ml. per brain and a final concentration of 0.015 *M* ACh. Immediately after the addition of ACh, a one-ml. aliquot was removed, subdivided into 0.2-ml. samples, and assayed for ACh by the colorimetric method of Hestrin (1949). After incubation for a total of one hour, a second one-ml. aliquot was withdrawn and the ACh measured as just described. The non-enzymatic hydrolysis of ACh was determined by the use of a 4.0-ml. blank containing all reagents except the homogenate. The ACh content of one-ml. aliquots of the blank was measured immediately after the ACh addition and also after one hour's incubation. This correction was applied in the calculation of the amount of ACh enzymatically hydrolyzed per hour.

Ammon's (1933) method of ChE assay was used more generally since a homogenate hydrolyzing as little as 0.05 μM of ACh per hour could be detected when 5-ml. vessels were employed. This greater sensitivity permitted determinations on a single brain, or on 3 or 4 ganglia. In performing the analysis the homogenate of brain or ganglion was diluted to 0.9 ml. with a solution containing 0.56 *M* NaCl and 0.079 *M* $NaHCO_3$. The resulting suspension was transferred to 5-ml. Warburg vessels and the manometers gassed with 95 per cent nitrogen and 5 per cent carbon dioxide for five minutes. After a further 10 minutes of temperature equilibration at 25° C., 0.1 ml. of 0.15 *M* ACh was tipped in from the side-arm of each vessel. Shaking was begun immediately and the first reading was taken 5 minutes later and continued at 10-minute intervals for 30 minutes. The thermobarometer contained all components in the reaction mixture except the homogenate; it therefore corrected for the non-enzymatic hydrolysis of ACh. The results obtained by the colorimetric and manometric methods were indistinguishable and the data have been treated as a unit.

The results were computed on a per brain or per ganglion basis, rather than in terms of weight of tissue. To make possible rough comparisons of the data, groups of equivalent brains and ganglia were excised, gently blotted on filter paper, and

weighed individually on a micro-torsion balance. The pupal brain weighed 1.22 ± 0.19 mg., and each ganglion, 0.19 ± 0.03 mg.

c. *Assay of cholinergic substances.* Brains or ganglia were dissected from unanesthetized animals and placed in one ml. of sea water containing 0.01 per cent eserine sulfate (Merck) and brought to pH 4 by the addition of dilute HCl. The excision required about 30 seconds for each brain and 30 to 60 seconds for ganglia. The extracts of 2 or 3 brains, or of 6 to 12 ganglia, were boiled for one minute, cooled, and stored at − 20° C. (Welsh, 1943). The isolated ventricle of the quahog, *Venus mercenaria,* was used in the assay of cholinergic substances, according to the method of Welsh (1943). About one-third of the assays were performed on ventricles which had been exposed for 15 minutes to 10^{-6} *M* LSD-25 (lysergic acid diethyl amide, Sandoz Products Ltd.). As Welsh (1954) has shown, this drug excites the molluscan heart maximally and thereby provides an excellent preparation for the assay of inhibitors of the ventricular beat. LSD treatment also masks the action of any excitors which might be present in the tissue extract.

d. *Choline acetylase.* In these determinations brain homogenates were incubated in a solution containing the salts, substrates, and cofactors favoring the acetylation of choline. The incubation media contained eserine to inhibit ChE, which would otherwise hydrolyze the desired product. The presence of fluoride is also necessary to protect the added ATP by inhibiting ATP-ases.

For each assay six animals at the same stage of development were anesthetized with carbon dioxide. The brains were excised and ground in a small, conical,

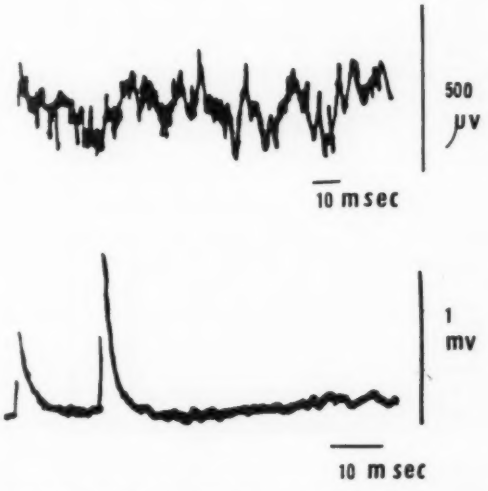

FIGURE 1. The electrical activity in the brain of a developing adult. The upper record was made with a fine stainless steel needle as the active electrode inserted into the brain and shows the summed potentials from many active neurons. The electrode for the lower record was an indium-filled micro-pipette with a tip diameter of approximately three microns. The action potentials of two individual neurons are shown.

glass homogenizer. The homogenate was diluted with 0.8 ml. of a solution containing the following reagents in the final concentrations specified: 18.7 mM choline chloride, 18.7 mM sodium acetate, 90 mM cysteine hydrochloride, 36.3 mM KCl, 0.9 mM $MgCl_2$, 1.9 mM Na_2HPO_4, 31.3 mM NaF, and 0.5 mM eserine sulfate; the pH was initially brought to 7.0 by the addition of HCl (Nachmansohn and Wilson, 1951; Gardiner and Whittaker, 1954). After equilibration of the reaction mixture in a water bath at 25° C., 0.1 ml. of 60 mM ATP.Na_2 (Pabst Labora-

TABLE I

The electrical activity of the brain during diapause and adult development

Stage of the animals	No. of observations	No. with active brains	No. with inactive brains
Platysamia cecropia			
Larvae—Days after beginning of cocoon construction			
0 days	4	4	0
2 days	5	5	0
5 days	5	5	0
7 days	5	5	0
9 days	3	0	3
10 days (Pupal moult)	5	0	5
Unchilled diapausing pupae	17	0	17
Diapausing pupae after storage at 6° C. for			
8 weeks	5	0	5
14 weeks	2	0	2
54 weeks	3	0	3
Pupae chilled for 8 weeks at 6° C. and then placed at 25° C. for 13 days	3	3	0
Pupae chilled for 10 weeks at 6° C. and then placed at 25° C. for 7 days	3	3	0
Pupae chilled for 13 weeks at 6° C. and then placed at 25° C. for			
0 days	3	0	3
2 days	2	0	2
4 days	2	0	2
6 days	3	2	1
8 days	3	3	0
During adult development			
0 day	2	2	0
1st day	2	2	0
2nd day	5	5	0
7th day	3	3	0
14th day	6	6	0
19th day	4	4	0
adult moth	4	4	0
Telea polyphemus			
Diapausing pupae	3	0	3
Developing adults	5	5	0
Samia walkeri			
Diapausing pupae	2	0	2
Developing adults	4	0	4

tories) and 0.1 ml. of a solution containing 360 Lipmann units of Coenzyme A (Pabst Laboratories) were added. After one hour incubation, dilute HCl was added to lower the pH to 4, and the mixture was boiled. The sample was then stored at − 20° C. and ultimately thawed and assayed on the *Venus* ventricle. The assay was controlled by identical determinations carried out on a mixture containing homogenate and all reagents except ATP.

Results

1. *The electrical activity of the central nervous system*

Figure 1 illustrates the electrical activity of the brain of the post-diapausing insect as recorded by two different techniques. The upper record was made with a stainless steel electrode, and shows the complex pattern of electrical activity generated by the "spontaneous" activity of many brain neurons. The second record, made with a microelectrode, shows the spontaneous activity of only two neurons. These records are typical of the electrical activity regularly detected in the brains of caterpillers and in the brains of animals after the end of diapause (Table I).

It is of special importance, as indicated in Table I, that no trace of activity could be recorded from the brains of diapausing pupae or of chilled pupae. This fact was confirmed, not only on Cecropia, but also on *Telea polyphemus* and *Samia walkeri*. Moreover, electrical stimulation of the ventral nerve cord or the subesophageal connective failed to evoke a response in the brain. Indeed, it was impossible to record any electrical response of the brain even when the brain was stimulated directly. Clearly most, and perhaps all, of the neurons of the diapausing brain are electrically inexcitable—the only uncertainty in this conclusion being the possibility that potentials from the smallest fibers of the insect brain were below the noise level of the amplification system. In summary, we see in the electrical measurements a profound difference between the brains of diapausing and post-diapausing pupae. This difference, in my experience, was clear cut: either the brain was electrically active or it was electrically silent.

As recorded in Table I, the electrical activity characteristic of the larval brain is already absent by the day before the pupal molt. The electrical silence then persists in pupae stored for as long as six months at 25° C. Indeed, the brains were still electrically inactive in pupae stored for a year at 6° C. Apparently, even prolonged chilling does not restore electrical activity to the brain. It will be noted in Table I that when pupae chilled for a long time were placed at 25° C. the electrical activity of the brain reappeared after only a few days. For example, in pupae stored at 6° C. for 13 weeks, the activity first appeared on the sixth day after transfer to 25° C. This corresponds to three days before the first visible signs of the onset of adult development.

It is of considerable interest that the electrical inactivity of the diapausing pupa is confined to the brain. Detailed electrical studies showed that all of the ganglia of the ventral nerve cord, from the subesophageal ganglion to the eighth abdominal ganglion, retained spontaneous activity throughout diapause, and that the ventral nerve cord always responded to electrical stimulation.

2. Cholinesterase in the central nervous system

The endocrinologically inactive brain of the diapausing pupa has been found to be electrically silent. Electrical activity disappears from the brain just before pupation. And the resumption of development at the end of diapause is prefaced by the reappearance of the brain's electrical activity. The electrical changes lead one to anticipate corresponding changes in the chemical mechanism underlying axonal conduction and synaptic transmission. For this reason the behavior of ChE was studied by the techniques described in the section on Methods.

As shown in Figure 2, the brains of fourth instar larvae contain a significant titer of ChE. ChE remains constant in the larval brain during the molt to the fifth instar and then slowly increases to the high titer characteristic of the brains of spinning caterpillars. After completion of the cocoon, the high titer of ChE per-

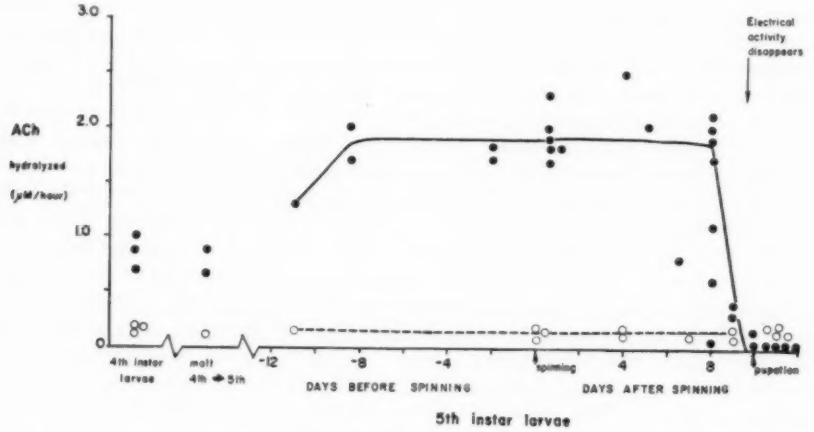

FIGURE 2. The cholinesterase activity of larval brains and ganglia. The ordinate shows the amount of ACh hydrolyzed per brain (or ganglion) in one hour. ●, brains; ○, ganglia.

sists in the brain for six days. Then, in the two days remaining before the pupal molt, the brain's ChE falls precipitously and becomes undetectable two days later. It is interesting that the fall in ChE is synchronous with the loss of the brain's electrical activity.

Figure 2 also shows that the ganglia of the ventral nerve cord maintain a uniform titer of ChE throughout the final larval instar and the pupal stage that follows. In short, the virtual absence of ChE is characteristic of only the brains of diapausing pupae. Of eight brains from fresh pupae subjected to individual analysis, only one showed any detectable ChE. Even this exceptional brain contained only a trace, corresponding to the hydrolysis of 0.07 μM of ACh per hour. Similarly, ChE was undetectable in individual determinations on three diapausing brains of *Telea* and on five brains from diapausing *Samia*.

Prolonged exposure of diapausing Cecropia to low temperature is without detectable effects in restoring the brain's ChE. Fourteen determinations on a total of 44 brains from pupae chilled at 6° C. showed that cholinesterase remained undetectable, even though two of the determinations were on the brains of pupae chilled for as long as 30 weeks. Consequently, it is clear that the diapausing brain, even after prolonged chilling, is characterized by a ChE titer undetectable by the present methods. In the preceding section these brains were likewise found to be electrically silent.

In the brains of previously chilled pupae ChE becomes detectable after a few days at 25° C. For example, Figure 3 illustrates results obtained on a series of pupae previously chilled for 13 weeks at 6° C. When the animals were placed at 25° C. and their brains analyzed at successive intervals, ChE remained undetectable for the first four days. Then, on the fifth day, brain ChE became barely detectable. Thereafter the activity increased rapidly until the onset of adult development, and then more slowly. Apparently, a slight but definite decline in ChE occurs prior to the emergence of the adult moth. Figure 3 likewise makes clear that the return of detectable titers of brain ChE correlates in time with the appearance of electrical activity.

Similarly, a total of six individual determinations on the brains of developing *Telea* and eight determinations on the brains of developing *Samia* showed that the post-diapausing brains of these species contained finite titers of ChE.

It will be recalled that the ventral nerve cord retains electrical activity throughout diapause. The ganglia from diapausing or chilled pupae also retained ChE, hydrolyzing an average of 0.13 ± 0.05 μM of ACh per ganglion per hour. As recorded in Figure 3, the enzymatic activity of the ganglia remains uniform throughout the entire course of adult development.

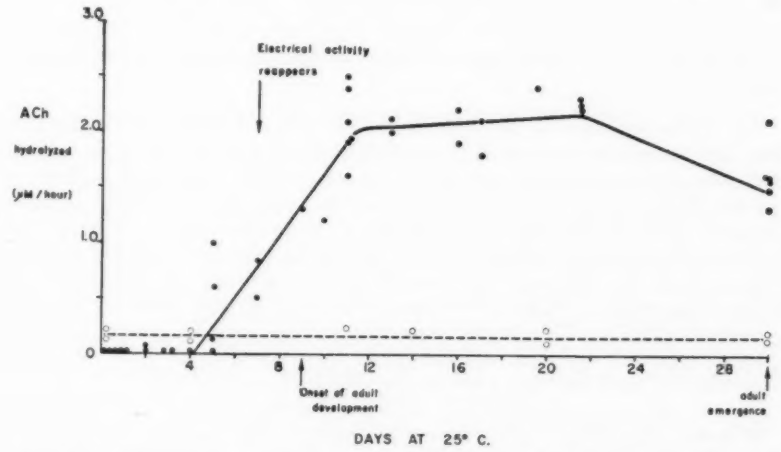

FIGURE 3. Cholinesterase activity in the brains and ganglia of pupae chilled for 13 weeks and then brought to 25° C. The ordinate shows the amount of ACh hydrolyzed per brain (or per ganglion) in one hour. ●, brains; ○, ganglia.

3. *The absence of a cholinesterase inhibitor*

In order to exclude the possibility that some inhibitor in the diapausing brain blocks the action of ChE, homogenates containing ChE were prepared, each containing the brains of two non-diapausing animals. The brei of a diapausing brain was added to half of each homogenate, and the ChE activities of both the remaining half of the original homogenate and the homogenate plus diapausing brain were measured. There was no evidence of inhibition in three experiments in which brains from newly diapausing pupae were added, or in three experiments in which chilled diapausing brains were added.

4. *Cholinesterase and electrical activity in the brain of a non-diapausing insect*

It seemed possible that the disappearance of electrical activity and of cholinesterase in the brain of the diapausing pupae might merely reflect a prolongation, by the intervention of diapause, of a sequence of events which normally occurs during the metamorphosis of non-diapausing species. To examine this possibility, the brain of *Galleria*, a non-diapausing lepidopteran, was studied. At 25° C. *Galleria* requires ten days from the pupal molt to the emergence of the adult moth; the true pupal stage is only the first 30 hours of this period (Schneiderman, 1952). Accordingly, electrical observations were made on the brains of six *Galleria* pupae selected during the first eight hours after the pupal molt. All of the brains were electrically active.

In further experiments, two *Galleria* were collected on each of the first six days after the pupal molt. The brains were excised and individually assayed for ChE. The titer of ChE activity corresponded to 0.9 ± 0.2 μM of ACh hydrolyzed per brain per hour; the activity showed no correlation with the stage of adult development.

5. *The effect of prothoracic gland hormone on the resynthesis of cholinesterase by the brain*

The results reported so far show that ChE is first detected in the pupal brain a few days before the onset of adult development; that is, at the beginning of the period when the brain's neurosecretory cells are probably maximally active. Consequently, it was important to decide whether the reappearance of brain ChE was a cause or a result of the recovery of the brain's endocrine action. The method, in brief, was to determine whether ChE appeared in previously chilled brains cultured *in vivo* in the absence of prothoracic gland hormone. To this end, a series of diapausing pupal abdomens were prepared from Cecropia pupae by the method of Williams (1947). Two weeks later, two or three pupal Cecropia brains were implanted into each abdomen and the preparation stored at 25° C. After two or four weeks the brain implants were removed and assayed for ChE.

The results, summarized in Table II, show that the synthesis of ChE took place in the previously chilled brains during the period of incubation in isolated abdomens. But unchilled brains under similar conditions were unable to synthesize ChE. Apparently, the synthesis of ChE is dependent on prior exposure to low temperature but is not dependent on the presence of the prothoracic gland hormone.

TABLE II

Cholinesterase in brains after incubation in isolated abdomens

Temperature treatment of the brain donors	Length of incubation in isolated abdomens	ACh hydrolyzed (μM/brain/hour)
Stored continuously at 25° C.	2 weeks	0
		0
		0
		0
		0
		0.2
Stored at 6° C. for 13 weeks	2 weeks	0.6
		0.7
		0.9
		1.0
Stored at 6° C. for 18 weeks	2 weeks	1.6
		1.8
Stored at 6° C. for 18 weeks	4 weeks	1.9
		2.0
		2.0

This conclusion was reinforced in a second series of experiments testing whether the brain hormone or the prothoracic gland hormone could promote the appearance of ChE in unchilled pupal brains. Brains from unchilled pupae were exposed to these hormones by implanting them into the tip of the abdomen in previously chilled pupae. The latter were then placed at 25° C. and allowed to begin adult development. On the second day of adult development the implanted brains were recovered and assayed for ChE. Five experiments performed on individual brains showed that the unchilled brains did not acquire detectable ChE when exposed in this manner to both the brain and prothoracic gland hormones.

6. *The cholinergic substance in brains and ganglia during diapause*

The remarkable variation in the ChE activity of the brain encouraged a study of the changes occurring in the corresponding cholinergic substrate during diapause and development. Results based on the *Venus* heart assay are summarized in Figure 4. Brain extracts from newly pupated animals showed only a trace of cholinergic activity. In marked contrast to the behavior of ChE, the brain's cholinergic activity undergoes a definite increase during diapause, the rate of increase being slow at 25° C. and rapid at 6° or 15° C. As mentioned in the section on Methods, the fact that about one-third of the *Venus* hearts had been treated with LSD-25 gives assurance that the changes are due to an increase in cholinergic substance rather than to a decrease in adrenergic substance.

As shown in Figure 5, the changes in the cholinergic substance during diapause are peculiar to the brain. The ganglia of the ventral nerve cord maintain a uniform titer of cholinergic substance during diapause, irrespective of the temperature of storage or of the duration of diapause.

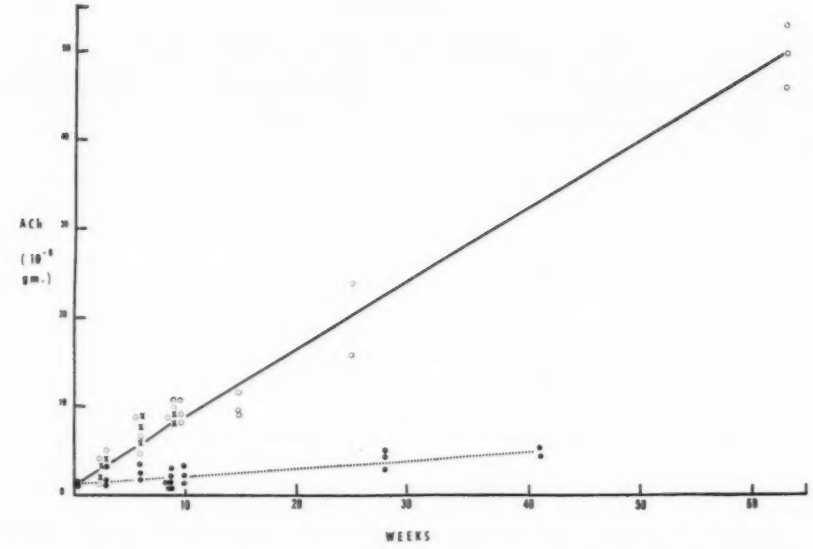

FIGURE 4. The titer of cholinergic substance in the brain as affected by the duration of diapause and the temperature or storage. The titer is expressed as the amount of ACh.Cl possessing identical action on the *Venus* ventricle. ○, stored at 6° C.; ×, stored at 15° C.; ●, stored at 25° C.

7. *The cholinergic substance during the reactivation of the brain*

In the preceding section the diapausing brain was found to acquire a considerable titer of the cholinergic substance during chilling. Figure 6 summarizes the history of the cholinergic material subsequent to the transfer of the chilled pupae from 6° to 25° C. Cholinergic material persists at high concentrations during the

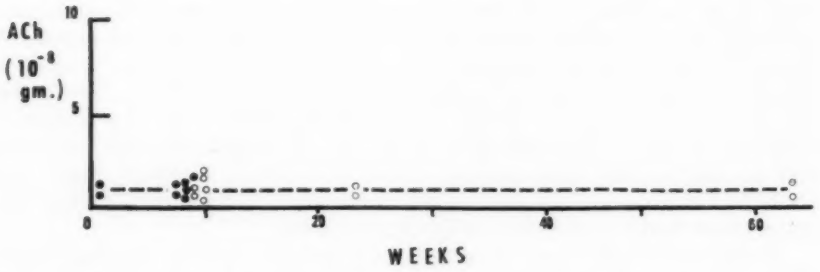

FIGURE 5. The titer of cholinergic substance in pupal ganglia as affected by the duration of diapause and the temperature of storage. Ordinate as in Figure 3. ○, stored at 6° C.; ●, stored at 25° C.

first few days at 25° C. and then undergoes a precipitous decrease. This sudden drop in cholinergic substance coincides in time with the reappearance in the brain of detectable ChE. The cholinergic substance then remains at the low and apparently constant level until just prior to adult emergence, and then increases fivefold to the high level characteristic of the adult brain.

Figure 6 also records corresponding measurements of the cholinergic substance in the ganglia of the same animals which served as donors of the brains. Here again, the changes are peculiar to the brain and are not encountered in the ganglia.

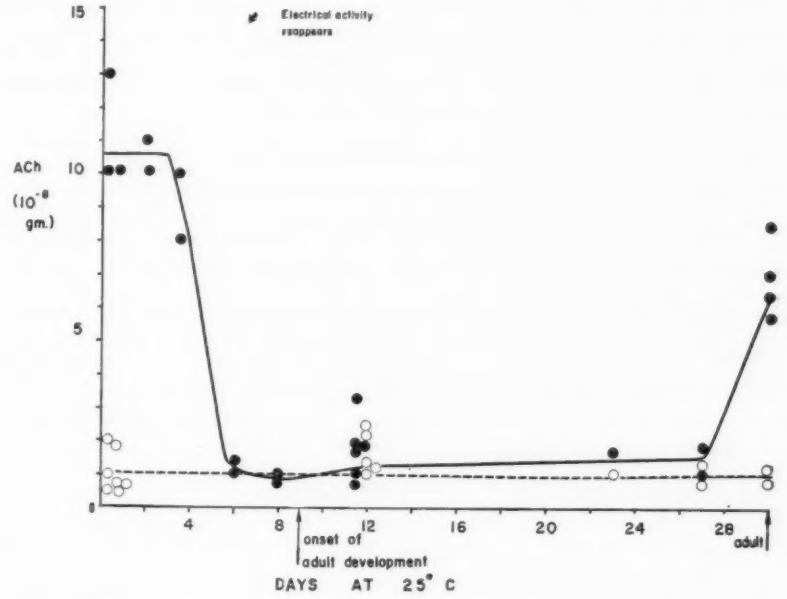

FIGURE 6. The titer of cholinergic substance in the brains and ganglia of pupae chilled for 13 weeks and then brought to 25° C. Ordinate as in Figure 3. ●, brain; ○, ganglia.

8. *The nature of the cholinergic substance*

Though the nature of the cholinergic substance has not been explored in detail, two observations show that the substance from Cecropia has certain resemblances to ACh. Cecropia brain extracts failed to depress the beat of *Venus* ventricles which had been previously treated with 0.01 per cent "Mytolon" [2:5-bis(3'diethylaminopropylamino)-benzoquinone bis-benzyl chloride, Sterling-Winthrop], a drug which has been shown by Welsh and Taub (1953) to block the action of ACh and related compounds by combining with the specific protein receptor in the *Venus* ventricle. Furthermore, the compound from Cecropia shares acetylcholine's

properties of being promptly destroyed by boiling at alkaline pH's, but resistant to boiling at acid pH's.

9. *Adrenergic substances in the Cecropia brain*

In view of the marked changes in the brain's cholinergic substance during diapause and development, there remained the possibility that molecules with an adrenergic action might also correlate with this change. Fortunately, as discussed above, "Mytolon" blocks the action of cholinergic substances on the *Venus* ventricle so that the heart after this treatment becomes suitable for the assay of adrenergic agents. A total of 18 pupal brains was prepared in six equal extracts and tested by this technique. Negative results were obtained in all cases, irrespective of the stage of the donors. The brain appears to contain no detectable adrenergic agent. However, the term "detectable" signifies very different concentrations of the various adrenergic agents: as high as 5×10^{-5} *M* for adrenaline, as low as 10^{-10} *M* for 5-hydroxytryptamine (Welsh, 1953).

10. *Choline acetylase in diapausing and developing brains*

Since the synthesis of ACh and perhaps of other cholinergic substances is catalyzed by choline acetylase, the changes observed in the brain's ACh might be secondary to changes in the choline acetylase. This possibility was explored by assaying the choline acetylase concentration according to the technique described under Methods.

The results, summarized in Table III, show that choline acetylase is present in

TABLE III

Choline acetylase activity in brains of animals at various stages

Stage of the animals	ACh synthesized ($\times 10^{-3}$ gm./brain/hour)
Unchilled diapausing pupae	0.6
	0.8
	1.2
	1.7
	3.1
Pupae after 9 weeks of storage at 6° C.	0.8
	1.5
	1.6
	1.7
	1.7
	2.1
Pupae chilled for 13 weeks at 6° C. and then placed at 25° C. for 8 days	1.3
	2.5
Zero day of adult development	1.0
	3.0
Second day of adult development	2.1
	6.2
	7.5
Seventeenth day of adult development	3.5
	6.2

the brain throughout pupal diapause and adult development. Furthermore, its titer in the pupal brain shows no correlation with the previous temperature history of the animal, or with the reappearance of ChE, or with the onset of electrical excitability. However, as Table III reveals, a prominent increase in the choline acetylase of the brain takes place after the onset of adult development.

Discussion

1. *The physiology of the brain during metamorphosis*

In Figure 7 the results of the present study are summarized and compared with the recurrent periods of endocrine activity of the brain as estimated from the studies of Williams (1946b, 1952, unpublished data) and Schneiderman and Williams (1953). The figure makes clear the parallel between the activity of the brain's neurosecretory cells and the physiology of the brain as a whole. It will be observed that the brain of the fifth instar caterpillar is endocrinologically active, electrically active, and contains a high titer of ChE. All of these parameters decline rapidly in the brain of the pre-pupa, so that by the time of the pupal molt the brain is endocrinologically inactive (Williams, 1952), electrically silent, and devoid of detectable ChE.

The electrical and enzymatic defects in the brain persist throughout the months of pupal diapause, and during this same period the brain is thought to be endocrinologically inactive. However, Williams (unpublished data) has recently shown that during prolonged chilling at 6° C. the neurosecretory cells apparently begin to release small quantities of brain hormone, though only after a year or longer at the low temperature does this "leakage" of hormone become sufficient to trigger adult development. In the present study the sole change found in the brain of the diapausing pupa was the progressive accumulation of cholinergic substance. This accumulation, as we have seen, is slow at room temperatures and rapid at low temperatures. The significance of this result will be discussed below.

As Figure 7 also shows, when a chilled pupa is transferred to 25° C. the brain is promptly reactivated. The first change detected is the appearance of measurable titers of brain ChE. Simultaneously, the cholinergic substance in the brain falls

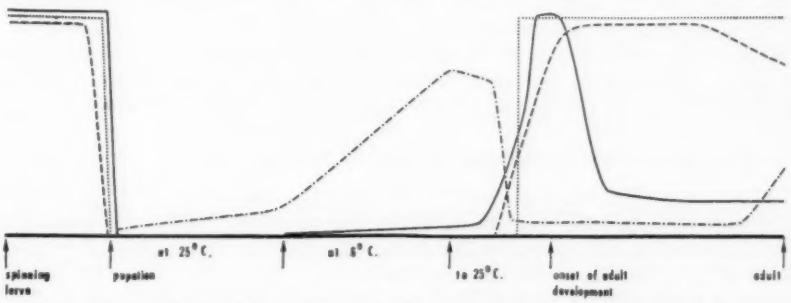

Figure 7. A diagrammatic summary of the physiology of the brain and the release of brain hormone during metamorphosis. The time scale during diapause is, of course, greatly condensed. For explanation see text. —, release of brain hormone; · · ·, electrical activity of the brain; — —, ChE in the brain; · —, titer of cholinergic substance in the brain.

precipitously from the high titer accumulated during chilling to the low titer characteristic of adult development. Immediately after the reappearance of ChE the brain regains electrical activity.

It is impossible to establish with the same precision the time when the neurosecretory cells begin to secrete the brain hormone. In the absence of a direct assay of the brain hormone it is necessary to infer the time of its release from indirect evidence. Schneiderman and Williams (1953) have estimated the time of release of prothoracic gland hormone. Their estimate is based on measurements of the oxygen consumption of previously chilled pupae during the interval between transfer to 25° C. and the onset of adult development. The results show that the oxygen consumption of such pupae begins a progressive increase about seven days before the onset of adult development. Schneiderman and Williams suggest that the increase in oxygen consumption results from the action of prothoracic gland hormone. Since the brain hormone is prerequisite for the function of the prothoracic glands, we are led to infer that the release of brain hormone begins more than seven days before the onset of adult development at 25° C. But in the present study the first changes in the physiology of the brain begin only five days before the onset of adult development. However, it is probably not prudent to emphasize this minor discrepancy between the data of Schneiderman and Williams and the present results, because the previous temperature histories of the pupae studied were quite different.

As Williams (1946b) demonstrated, the brain's neurosecretory cells are maximally active only until the first days of adult development. Activity then declines. Originally it was believed that at this time the neurosecretory cells lost all activity, but in recent extensive studies Williams (personal communication) found that in the adult brain the neurosecretory cells may maintain a low rate of hormone release. By contrast, the brain's ChE progressively increases from the time of reappearance until a maximum is reached on the second day of adult development. The titer is maintained until shortly before the emergence of the adult moth, when a decrease in brain ChE takes place. On the other hand, the cholinergic substance in the brain remains at a uniform low titer throughout adult development and finally increases five-fold shortly before the emergence of the adult moth. These changes, as we have seen, are without effect on the brain's electrical activity, which continues unaltered during adult development.

It is worth recalling that all of the changes shown in Figure 7 are peculiar to the brain. Throughout metamorphosis the ganglia of the ventral nerve cord retain electrical activity, ChE, and a uniform titer of cholinergic substance.

2. *Cholinesterase and the electrical activity of the brain*

The results show that the titer of ChE during metamorphosis is paralleled by changes in the electrical activity of the brain. At pupation, ChE becomes undetectable and the brain becomes electrically silent. Months later, the return of detectable ChE is followed by the return of electrical activity to the brain. This striking correlation is interesting in view of the considerable evidence, summarized by Nachmansohn (1953–54), that ChE is essential for conduction in axons. In any event it seems probable that the disappearance of ChE can, in itself, account for the loss of electrical activity of the Cecropia brain.

It is possible, of course, that the diapausing brain loses enzymes in addition to ChE. However, the results show that choline acetylase, an enzyme of the same metabolic cycle, persists in the brain throughout diapause; indeed, the titer of choline acetylase appears to remain unchanged as electrical activity returns to the brain. At present there is no reason to suppose that diapause is accompanied by profound changes in any of the brain's enzymes except ChE.

3. *Electrical activity and brain hormone release*

Since no direct assay is known for the brain hormone, it is difficult to correlate precisely the electrical activity and the release of brain hormone. At the onset of diapause the endocrine competence and the electrical activity of the brain are lost simultaneously. Here the electrical inactivity of the brain can account for the failure of neurosecretion and the consequent onset of diapause. At the end of diapause the situation is less conclusive. It seems clear from the available facts that the rapid release of brain hormone before the onset of adult development occurs at about the time when the brain regains electrical activity.

These inferences are supported by the investigations of the causes of the reappearance of ChE in the brain. It will be recalled that previously chilled brains were able to synthesize ChE, even when cultured in the absence of the prothoracic gland hormone. Unchilled brains under the same circumstances were unable to synthesize ChE even when exposed to the brain and prothoracic gland hormones. Williams (personal communication) has already shown that unchilled pupal brains fail to regain endocrine competence when exposed to brain and prothoracic gland hormones. Apparently, the factors which promote the return of ChE to the brain are precisely the same as the factors which elicit the return of endocrine activity.

4. *The changes in the brain during diapause*

The results show that the reappearance of detectable ChE and of endocrine activity is hastened by a few weeks of exposure to low temperatures. The low temperatures favor the rapid accumulation of a cholinergic substance in the pupal brain. When a high titer of cholinergic substance is present in the brain, the neurosecretory cells are promptly reactivated when returned to 25° C. The analysis so far suggests that the reactivation of the neurosecretory cells depends on the synthesis of ChE and the consequent establishment of electrical activity in the brain as a whole. It therefore seems possible that the high titer of cholinergic substance triggers the prompt synthesis of ChE; that is, the synthesis of ChE may be an adaptive response to high substrate concentrations. As Featherstone *et al.* (1955) have shown, the adaptive synthesis of ChE can be elicited in chick intestine cultivated *in vitro*.

The evidence suggests that the pupal diapause of Cecropia results from the failure of the brain as a whole. The diapausing brain is electrically silent, apparently because of the absence of ChE. Evidently, it is essential for the pupa to inactivate the entire brain to insure the persistent inactivity of the neurosecretory cells. ChE is slowly synthesized by the brain after prolonged storage at room temperatures, but promptly following storage at low temperatures. Low temperatures favor the rapid accumulation of a cholinergic material in the brain which suggests

that the reappearance of ChE is an adaptive response to high substrate concentrations. When ChE reappears, the brain becomes electrically active, the neurosecretory cells release brain hormone, and the long season of diapause is ended.

Professor Carroll M. Williams, of Harvard University, aided this investigation by sharing his unpublished observations, by many helpful discussions, and by a critical reading of the typescript of this paper. It is a pleasure to acknowledge my gratitude. I am also indebted to Professor J. H. Welsh, of Harvard University, for advice on ACh assay techniques and for providing several of the drugs used in the assays.

Summary

1. The 26 neurosecretory cells in the brain of the Cecropia silkworm have been shown by Williams to undergo systematic changes in endocrine activity during metamorphosis. At the pupal molt the neurosecretory cells lose endocrine activity. Consequently, growth is checked and the animal enters pupal diapause. Endocrine activity is restored following prolonged exposure to low temperatures. When the brain hormone is released, diapause ends and the development of the adult moth begins.

2. The electrical events in the brain as a whole have been studied in relation to these changes in endocrine activity. The larval brain shows abundant "spontaneous" electrical activity. However, on the day before the pupal molt the brain loses spontaneous activity and is no longer excited by direct electrical stimulation. The electrical silence of the brain persists throughout the months of diapause. Electrical activity reappears in the pupal brain a few days before the end of diapause.

3. Cholinesterase (ChE) is present in high titer in the larval brain. However, two days before pupation the brain ChE begins a rapid decline, so at pupation ChE is undetectable in the brain. ChE does not reappear in the pupal brain until shortly before the recovery of electrical and endocrine activities. There is no evidence that an inhibitor of ChE is present in the diapausing brain. Evidently, ChE is absent or in low titer and is synthesized just before the onset of adult development.

4. During diapause the cholinergic action of brain extracts steadily increases. The increase is slow in the brains of pupae stored at 25° C. and rapid in the brains of pupae stored at 15° or 6° C. Therefore the rate of accumulation of the cholinergic substance parallels the effectiveness of the temperature treatment in restoring the brain to endocrine activity.

5. When ChE reappears in the brain, just before the onset of adult development, the titer of cholinergic substance in the brain falls to a low level. The low level is maintained until shortly before adult emergence, when the titer of cholinergic substance increases five-fold.

6. Choline acetylase activity remains uniform in brain homogenates from unchilled and chilled pupae.

7. The changes in electrical activity, ChE, and cholinergic substance during metamorphosis are peculiar to the brain. The ganglia of the ventral nerve cord retain normal function throughout diapause.

8. ChE is synthesized in chilled pupal brains which are cultured at 25° C. in the absence of prothoracic glands. Under the same circumstances unchilled brains are unable to synthesize ChE. Moreover, exposure to the brain and the prothoracic gland hormones fails to promote the synthesis of ChE in unchilled pupal brains.

9. ChE and electrical activity remain constant during the metamorphosis of *Galleria*, a non-diapausing insect. Two additional diapausing species, *Telea polyphemus* and *Samia walkeri* showed variations in brain ChE and electrical activity identical to those described in Cecropia.

10. The evidence suggests that the pupal diapause of Cecropia results from the failure of the brain as a whole. The diapausing brain is electrically silent, apparently because of the absence of ChE. During diapause there is an accumulation of a cholinergic substance in the brain—an observation which suggests that the reappearance of ChE is elicited by high substrate concentrations. When ChE reappears, the brain becomes electrically active, the neurosecretory cells release brain hormone, and diapause is ended.

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ADENOSINETRIPHOSPHATASE OF MYTILUS SPERMATOZOA. I. EFFECTS OF pH, CALCIUM AND MAGNESIUM, AND CONCENTRATION OF ENZYME AND SUBSTRATE

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The flagellum is the motile organelle of the sperm cell. Motility is apparently independent of the immediate control of the head. Inactivation of the nucleus, as by the action of dyes on frog sperm, causes no damage to the extranuclear parts, and eggs inseminated with the treated sperm may go on to develop haploid embryos (Briggs, 1952); waving movements of sperm tails detached from heads have also been observed (Rothschild, 1953). It is, therefore, not surprising that an enzyme capable of splitting ATP has been found concentrated in the flagella of the sperm of certain marine invertebrates and of the bull (Nelson, 1951, 1954a).

Investigations into the nature of the enzyme associated with movement have included a variety of studies in other biological systems. These range from the effects of ATP on contraction of isolated bacterial flagella (DeRobertis and Franchi, 1952), on pseudopod formation and protoplasmic streaming in ameba (Goldacre and Lorch, 1950), and on rate of ciliary activity of frog pharyngeal mucosa (Vorhaus and Deyrup, 1953) to the effects of the cation composition and pH of the incubation medium on ATP dephosphorylation by vertebrate and insect muscle ATP-ase. A calcium-activated ATP-ase has been derived from the myosin component of muscle fibrils (Banga and Szent-Györgyi, 1943), as distinguished from a soluble magnesium-activated ATP-ase readily extractable from both vertebrate (Kielley and Meyerhof, 1948) and insect muscle (Gilmour and Calaby, 1952; Maruyama, 1954). Combination with actin has been found to reverse magnesium-inhibition, as well as to alter the pH optimum, of myosin ATP-ase (Sarkar et al., 1951).

The function of ATP in spermatozoa has been examined by a number of investigators (*cf.* reviews by Mann, 1949, 1954), all of whom conclude that ATP plays an important role in sperm motility. Depletion of the ATP content of semen coincides with impairment and cessation of motility of ejaculated bull spermatozoa (Mann, 1945). The sperm tail enzyme, tentatively referred to as adenylpyrophosphatase, preferentially hydrolyzed ATP, and was relatively inactive toward yeast adenylate, fructose diphosphate and beta-glycerophosphate (Nelson, 1954a). Apyrase may be extracted from bull sperm tails with glass-distilled water or 0.6 *M* KCl. While the KCl extracts nearly all of the enzyme, a high degree of activity remains with the residue of the water-extracted material. On the other hand, Mytilus sperm tails resist extraction by either of these methods. However, dilution of KCl or sea water suspensions with large quantities of ice-cold distilled water leads to agglutination and precipitation of the Mytilus sperm tails (Nelson, 1954b).

[1] With the technical assistance of Abolghassem Amin and Anne Ruth Nelson.

In order further to characterize the nature of this enzyme, studies have been conducted on the influence of pH, calcium and magnesium, and enzyme and substrate concentration on the rate of ATP dephosphorylation by Mytilus sperm tail preparations.[2] The results of these experiments are presented in this paper.

Materials and Methods

The testes of three or four bay mussels, *Mytilus edulis*, were excised and minced in a small volume of filtered sea water (FSW), and the sperm was separated from the mince by washing gently through four layers of cheese cloth. The sperm suspension was concentrated at a relative centrifugal force of 2000 × gravity for five minutes. The supernate was decanted and discarded and the sperm resuspended in 10 volumes of FSW; the washing is repeated several more times in either FSW or *M*/2 KCl. The spermatozoa may be "decapitated" by either of two methods: a) the sperm are placed in a chilled Pyrex mortar in the deep freeze until the suspension acquires a slushy consistency, at which time the suspension is ground with a Pyrex pestle for two minutes; or b) the suspension is diluted with FSW and run through a Logeman stainless steel hand homogenizer. Spinning the homogenate at 1000 × g for ten minutes separates the heads from the tails, which remain in the supernate. The residue may be re-suspended two more times in FSW or isotonic KCl and the centrifugation repeated, thereby increasing the recovery of the tails. The supernates are then pooled and the tails collected by centrifuging at 7000 × g for ten minutes. The supernate is discarded and the tails are then suspended in *M*/2 KCl. (All these operations are carried out in the cold room, or with equipment which has been chilled in the refrigerator, and ice-cold solutions employed.)

Phosphate liberation is measured according to Moog and Steinbach (1945) using a modification of the colorimetric method of Taussky and Shorr (1953) in the Klett-Summerson colorimeter with the No. 66 filter. The reactants are mixed in 12-ml. Pyrex conical centrifuge tubes and equilibrated in the thermostat at 26–27° C. for at least 5 minutes prior to the initiation of the experiment by the addition of the ATP. Each tube contained 0.2 ml. sample, and, in final concentrations, 0.05 *M* KCl, 0.01 *M* veronal buffer, 8×10^{-4} *M* or 1.6×10^{-3} *M* ATP[3] (0.1 or 0.2 mg. 7′-phosphate) plus glass-distilled H_2O and $MgCl_2$ or $CaCl_2$ to make a total volume of 2.0 ml. After incubation for 10 minutes in the thermostat, the reaction was terminated by the addition of 2.0 ml. ice-cold 10% trichloroacetic acid, the precipitate removed by centrifugation, and the entire supernate analyzed for orthophosphate. Reagent blanks are run in duplicate tubes to which the trichloroacetic acid was added prior to the ATP. Controls for the experiments on the influence of divalent cations included all the components of the reaction mixture except the magnesium or calcium chlorides, and were also run simultaneously with the experimentals. In these determinations, the results are expressed as per cent of the control phosphate liberation. In the studies on the influence of pH, and the effect of enzyme and substrate concentration, the results are stated in terms of specific activity, micrograms of phosphorus liberated per milligram of nitrogen per minute (N determined by the semi-micro-Kjeldahl method of Ma and Zuazaga, 1942).

[2] This work was partially supported by grants from the University of Nebraska Research Council.

[3] Disodium ATP (Pabst), neutralized with NaOH.

RESULTS

Effects of the divalent cations. The mode of preparation of the sperm affects the response of the tail enzyme to the divalent cations, calcium and magnesium. This is illustrated in Figure 1, where the enzyme activity of preparations washed 3 times in FSW and once in *M*/2 KCl is compared with that of sperm washed twice in FSW and 4 times in *M*/2 KCl. When the first washing procedure is followed,

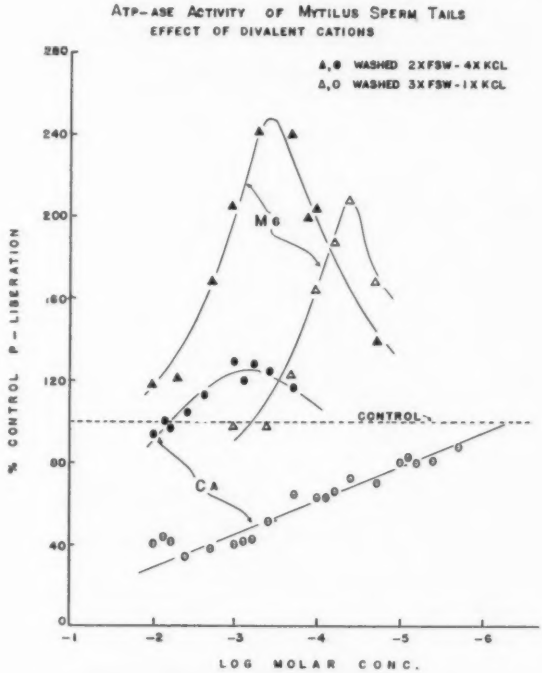

FIGURE 1. ATP-ase activity of Mytilus sperm tails. Effect of divalent cations. Sperm washed twice in filtered sea water (FSW) and 4 times in *M*/2 KCl ▲, ●. Sperm washed 3 times in FSW and once in *M*/2 KCl △, ⊙. Varying concentrations of $MgCl_2$ ▲, △. Varying concentrations of $CaCl_2$ ●, ⊙. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 8.4, 8×10^{-4} *M* ATP, $MgCl_2$ or $CaCl_2$, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 27° C. Ordinate—% control P-liberation, abscissa—log molar concentration divalent cation.

calcium inhibits in all concentrations, while the ATP-ase activity is maximal in 4×10^{-5} *M* $MgCl_2$. Not only is the optimum for magnesium shifted to a higher concentration, but calcium also now has a slightly activating effect on the enzyme when the latter method of washing is employed. Apparently a considerable amount of the divalent cations of the sea water remains with the residue when the sperm are washed only once in isotonic KCl after repeated washing in FSW. Since Woods

Hole sea water contains somewhat over 50 millimoles of magnesium, and about 9 millimoles of calcium per liter, this suggests that the residual cations following the single KCl wash not only contribute measurably to the ionic composition of the incubation medium, but also they exert mutually antagonistic effects on the enzyme ac-

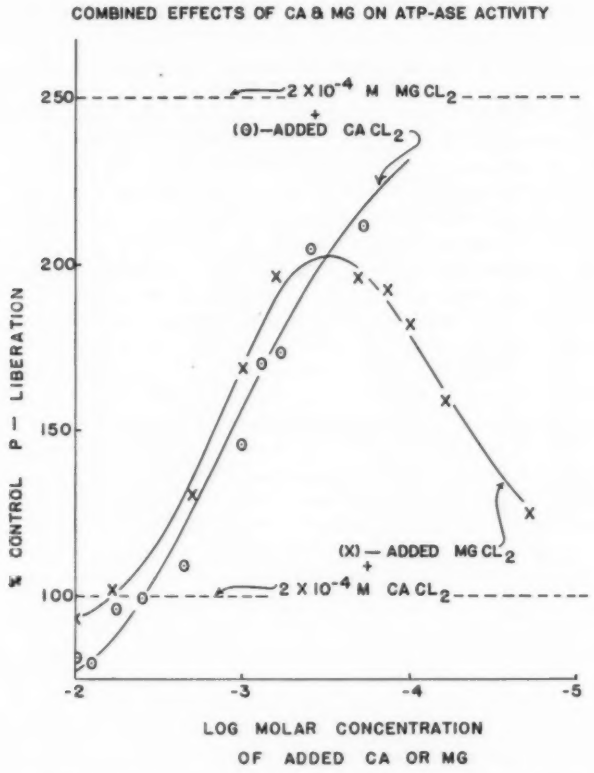

FIGURE 2. Combined effects of Ca and Mg on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M*/2 KCl. ⊙—$MgCl_2$, 2×10^{-4} *M* plus graded amounts of $CaCl_2$. ×—$CaCl_2$, 2×10^{-4} *M* plus graded amounts of $MgCl_2$. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 8.4, 8×10^{-4} *M* ATP, $MgCl_2$ as indicated, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 27° C. Ordinate—% control (no divalent cation) P-liberation, abscissa—log molar concentration of Ca or Mg added in graded amounts to reaction mixture containing fixed concentration of other divalent cation.

tion (an alternative mechanism is also indicated on the basis of the work of Tyler, 1953, that addition of amino acids, peptides, proteins and metal-chelating agents is beneficial to sperm by inducing complex formation with various toxic heavy metals commonly present in sea water). The present hypothesis may be tested on the 2 ×

FSW-4 × KCl wash preparations by adding varying amounts of one of the divalent cations to the reaction mixture containing a fixed concentration of the other. The results of such an experiment are summarized graphically in Figure 2. The fixed concentration of the cations was arbitrarily set at 2×10^{-4} *M*, since this was within the range of optimum activation by $MgCl_2$, and at this concentration the rate of ATP dephosphorylation in $CaCl_2$ was essentially the same as that of the control (no added divalent cation). All additions of Ca depressed the activation by 2×10^{-4} *M* $MgCl_2$; whereas the maximum antagonistic effect of Mg added to 2×10^{-4} *M* $CaCl_2$ obtained when the $MgCl_2$ reached about 3×10^{-4} *M*. Apparently the dif-

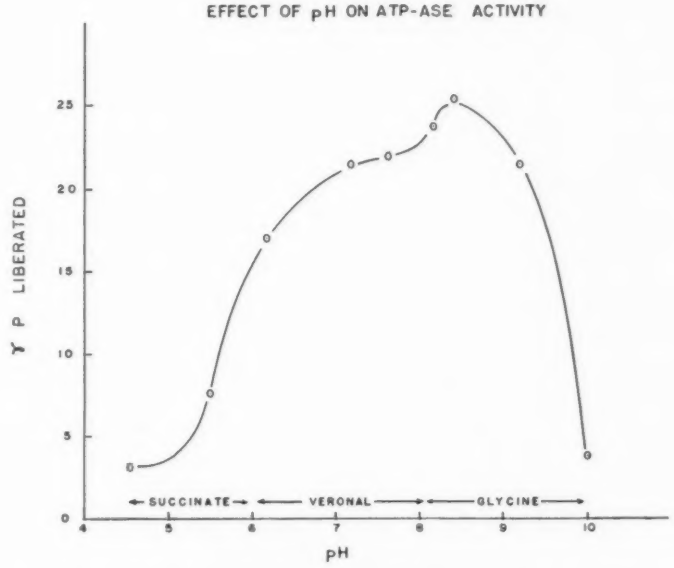

FIGURE 3. Effect of pH on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M*/2 KCl. Tube contents: 0.05 *M* KCl, 0.02 *M* succinate, veronal or glycine buffer, 0.02 *M* NaCl, 8×10^{-4} *M* ATP, 5×10^{-4} *M* $MgCl_2$, 0.2 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 26° C. Ordinate—P liberated/mg. N/min., abscissa—pH.

ference in the influence of the two divalent cations is not merely a matter of degree. Magnesium may potentiate the slightly stimulatory effect of calcium, while calcium in all concentrations antagonizes the magnesium effect.

Effect of pH. Three buffers were used covering the range from pH 4 to pH 10 in determining the optimum for liberation of phosphate from ATP by the sperm tail enzyme: succinic acid-sodium succinate, pH 4–6; HCl-sodium veronal, pH 6–9; glycine-NaOH, pH 8–10 (all measurements made with a glass electrode). In these experiments the ionic strength of the incubation medium was adjusted by the addition of appropriate amounts of NaCl. The enzyme activity rises gradually to

a fairly sharp peak in both veronal and glycine buffers at about pH 8.4, and drops off precipitously to pH 10 (see Fig. 3). This is of interest inasmuch as while sperm respiration is optimal at about pH 7, and below this both motility and metabolism progressively decline, alkalinity, up to pH 8.5 and above, enhances movement (Lardy and Phillips, 1943).

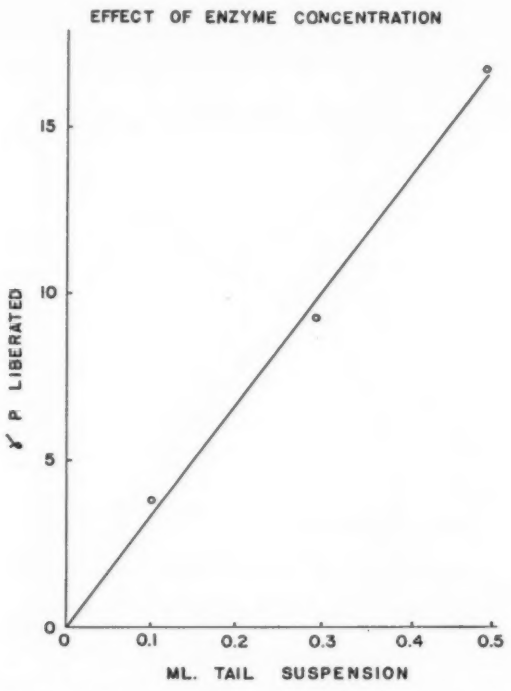

FIGURE 4. Effect of enzyme concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M*/2 KCl. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 8.5, 8×10^{-4} *M* ATP, 5×10^{-4} *M* $MgCl_2$, 0.1, 0.3 or 0.5 ml. sperm tail preparation; total 2.0 ml. Incubation: 10 min., 26.5° C. Ordinate—P liberated; abscissa—ml. tail suspension.

Effect of enzyme and substrate concentration. The proportionality between enzyme concentration, in terms of ml. of tail suspension, and rate of dephosphorylation of ATP is shown in Figure 4. The rate of phosphorylysis may also be shown to be a function of the ATP concentration, although in higher concentrations, the relationship apparently ceases to be linear (Fig. 5). Since approximately only 50% of the labile (7')-phosphate is liberated, even on prolonged incubation (Table I and Fig. 6), it may furthermore be concluded that the tail enzyme is a true adenosinetriphosphatase according to the definition of Steinbach and Moog (1945).

Discussion

It has previously been shown that an enzyme, concentrated in the flagellar components of spermatozoa, and either lacking or present only in minute amount in the head, is capable of dephosphorylating adenosinetriphosphate (Nelson, 1951, 1954a). Evidence has been presented establishing this as an adenosinetriphosphatase by virtue of the fact that it liberates only the terminal phosphate of ATP with appreciable velocity (Table I) under the conditions of the experiments. The fact that ATP not only plays an important role in, but is also apparently essential for,

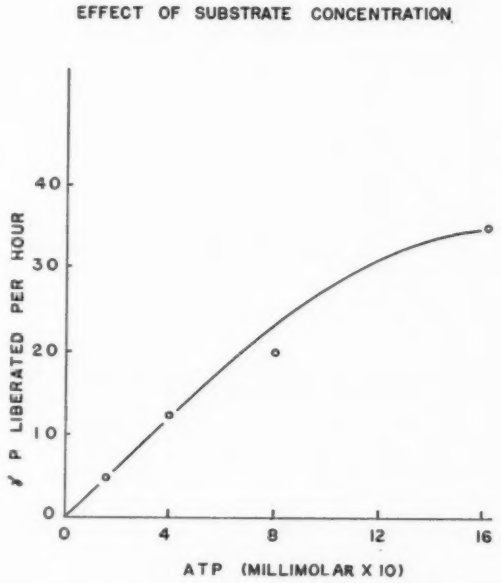

Figure 5. Effect of substrate concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M/2* KCl. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 9.2, 5×10^{-4} *M* $MgCl_2$, ATP conc. as indicated; 1.0 ml. sperm tail preparation; total 10.0 ml. Incubation: 1 hour, 27° C. Ordinate—γP liberated per hour; abscissa—ATP concentration.

sperm motility suggests that this spermatozoan ATP-ase may have other properties in common with the ATP-ase of muscle.

The splitting of ATP by actomyosin solutions is inhibited by a wide range of Mg concentration, the degree of inhibition being a function of the Mg/Ca ratio (Braverman and Morgulis, 1948). Extruded actomyosin (myosin B) threads split ATP, the rate of phosphorylysis being enhanced by $MgCl_2$, while $CaCl_2$ caused an even greater activation (Bowen, 1952a). On the other hand, in low concentrations of ATP, $MgCl_2$ greatly accelerates, and $CaCl_2$ strongly inhibits shortening of the threads (Bowen, 1952b). Glycerol-extracted muscle fibers in 0.1 *M* KCl never

reach maximum contraction in the absence of Mg ions, and the enhancing effect of calcium on the ATP-ase activity of these fibers is less than half that of magnesium, the optimum occurring at pH 7 (Sarkar, Szent-Györgyi and Varga, 1951). However, at KCl concentrations high enough to cause dissociation of the actomyosin complex in the presence of ATP, the glycerinated muscle has the same ATP-ase properties as myosin, *i.e.*, alkaline pH optimum and Ca ion activation. Calcium in-

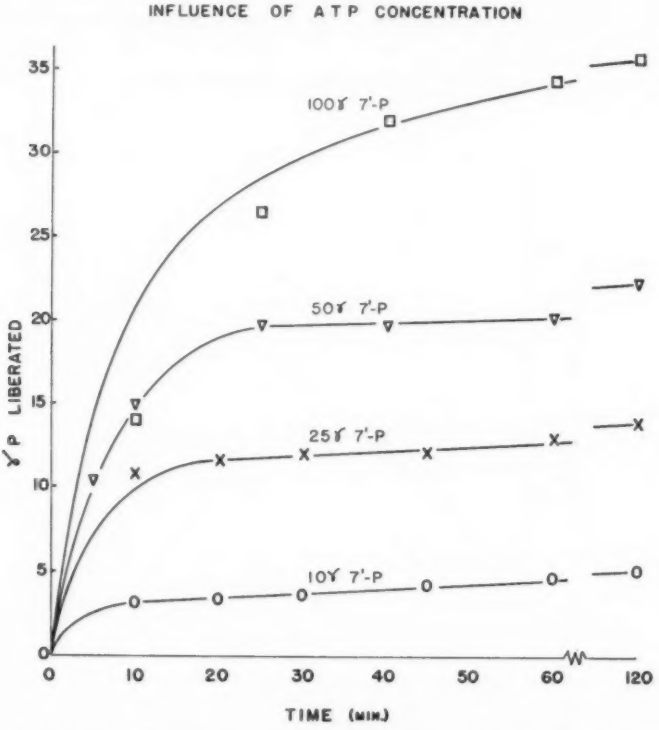

FIGURE 6. Influence of ATP concentration on ATP-ase activity of Mytilus sperm tails. Sperm washed twice in FSW, 4 times in *M*/2 KCl. Tube contents: 0.05 *M* KCl, 0.01 *M* veronal buffer pH 9.2, 5×10^{-4} *M* $MgCl_2$, ATP conc. indicated as γ7'-P, 1 ml. sperm tail preparation; total 10.0 ml. Incubation: 2 hours, 27° C. Aliquots tested at times indicated. Ordinate—γP liberated, abscissa—time in minutes.

duces shortening when injected into isolated single muscle fibers (Heilbrunn and Wiercinski, 1947); magnesium ions are about 1/70 as potent (Woodward, 1948). Hayashi (1952) has reported that pH 7.4–7.6 is optimum for contraction of pellicular actomyosin fibers, while Kafiani and Engelhardt (1953) noted a shift in the optimum to pH 9 for pellicular myosin fiber contraction, conditions most favorable for the ATP-ase activity of actin-free myosin. It has been pointed out that pro-

TABLE I

Effect of substrate concentration on hydrolysis of ATP

Tube contents: NaCl 7×10^{-3} *M*, $MgCl_2$ 3×10^{-4} *M*, veronal buffer 10^{-2} *M* pH 9.2, ATP, sperm tail prep. 1 ml.; total 10 ml.; temp. 27° C.

ATP conc. mM	Time min.	% labile P hydrolyzed	Rate γP/min./mg. N
0.16	10	28.8	3.2
	30	36	1.3
	60	45	0.8
	137	50.4	0.4
0.4	10	44	12.3
	30	48.5	4.5
	60	51.2	2.4
	120	55.5	1.3
0.8	10	29.6	16.5
	25	39	8.7
	60	40.4	3.8
	120	44.0	2.1
1.6	10	14.1	15.8
	25	26.5	11.8
	60	34.4	6.4
	120	35.7	3.3

nounced calcium activation of muscle apyrase follows depression of activity occasioned by extraction of muscle with strong KCl solutions, while hydrolysis of ATP by whole muscle homogenates is not activated by calcium (Steinbach, 1947). When frozen-ground muscle extracts are dialyzed against 0.1 *M* KCl, calcium shows a strong inhibition at all concentrations while small amounts of magnesium activate ATP hydrolysis.

Thus with respect alone to the effects of pH and ion composition of the incubation medium on the activity of crude sperm tail preparations, it is difficult directly to compare the sperm tail enzyme with muscle ATP-ase. However, it may be tentatively concluded that the alkaline optimum puts the sperm tail ATP-ase in the class of myosin or of glycerinated fibers treated with strong KCl solutions, while the enzyme activation in the presence of magnesium salts is similar to the behavior of glycerinated fibers and whole muscle homogenates in weak KCl solutions. Moreover, the precipitation of the Mytilus sperm tails from KCl suspension by dilution with large volumes of water is analogous to the dilution-precipitation of myosin particles from myosin sols.

It is pertinent to consider the relationship of some of these properties of the enzyme to the behavior of the spermatozoa in response to the various factors tested. As mentioned above, the effect of pH on the ATP-ase activity of the Mytilus sperm tails seems to parallel the effects on both metabolism and motility, at least of mammalian spermatozoa. The hydrogen ion concentration of fresh samples of sea urchin semen was found to be pH 7.6–7.9 for *Arbacia punctulata*, and pH 7.5 for *Echinus esculentus* (Hayashi, 1945; Rothschild, 1948).

In some preliminary observations, it was noted that the spermatozoan ATP-ase, like that of muscle (Szent-Györgyi, 1951), is relatively indifferent to the monovalent cation, essentially the same degree of enzyme activity occurring with either K or Na solutions; although addition of K ions to a suspension of motile trout sperm in NaCl inhibits their movement (Schlenk, 1933), Mytilus sperm remain motile even after washing in isotonic KCl. Although the magnesium content of mammalian semen is only one-half to one-third of the calcium content in mg% (Mann, 1954) the omission of Mg^{++} from diluents or extenders depresses metabolism and motility, while the addition of calcium adversely affects motility, glycolysis and respiration (Lardy and Phillips, 1943).

Summary

1. The tails of the sperm of *Mytilus edulis* dephosphorylate ATP.

2. $MgCl_2$ (5×10^{-4} *M*) increases the rate of the enzyme activity in 0.02 *M* KCl to more than double that of the control; the same concentration of $CaCl_2$ causes about 25% greater activity than the control.

3. In appropriate concentrations magnesium potentiates the effect of calcium, but all concentrations of calcium tested depress the magnesium activation.

4. The enzyme activity rises gradually to a fairly sharp peak at pH 8.4 in veronal and glycine buffers and drops off abruptly to pH 10.

5. The rate of dephosphorylation of ATP is proportional to the enzyme concentration.

6. Phosphorylysis is also a function of the ATP concentration, except at higher levels of ATP.

7. This is apparently a true ATP-ase since only about 50% of the labile phosphate is liberated with appreciable velocity from ATP.

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INFECTION OF COCKROACHES WITH HERPOMYCES (LABOULBENIALES). IV. DEVELOPMENT OF H. STYLOPYGAE SPEGAZZINI [1, 2]

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Although some 1500 species of Laboulbeniales have been described, the development of only a few species of relatively simple structure has been worked out in detail (Benjamin and Shanor, 1950). For the genus *Herpomyces,* Thaxter (1931) gives a short description of the general features of development, presumably pieced together from stages noted during the description of some two dozen species. But nowhere is there an illustrated description of the development of any species of *Herpomyces,* or, indeed, of any member of the Laboulbeniales showing this much structural complexity. The present paper gives a pictorial presentation of the development of both female and male plants with special reference to the differences when growth is on setae in contrast to the general antennal surface, and to the origin of multiple perithecia and development of the peculiar basal "shield" covering the secondary receptacle in this species.

In contrast to *Stigmatomyces* (Thaxter, 1896) and *Laboulbenia* (Benjamin and Shanor, 1950), the development of *Herpomyces stylopygae* is less precise. There is more variation in relative cell sizes and absolute cell number. The general picture, however, follows a regular pattern of development determined by sex and location on the host. And, in contrast to the dioecious *Laboulbenia formicarum,* the cell lineage in male and female plants is entirely different instead of nearly the same up to the time of differentiation of the reproductive organs.

Materials and Methods

Many of the specimens in our colony of the oriental cockroach (*Blatta orientalis* L.) are infected with *H. stylopygae,* and the fungus continues to maintain itself in these colonies. Since the antennal infections can be seen with the unaided eye, infected individuals were caught, the antennae amputated, prepared as whole mounts in lacto-phenol plus cotton blue, and examined. For older stages it is convenient to scrape the antennae and then study the dislodged plants floating freely in the stain medium. Younger stages have to be examined on the surface of whole antennae or on setae.

Serial sections of infected antennae were available from the study presented in Part 2 of this series; they were useful for certain points in later development when

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[2] The work reported in this paper was done in connection with a contract between the Office of the Surgeon General, U. S. Army, and the University of Minnesota.

the plant had become too large for satisfactory examination as a whole mount (Figs. 26–29, 44).

The method of drawing particular stages and then attempting to seriate the drawings has distinct limitations. Yet it is not feasible to follow the development of a single plant when they can be grown only on living cockroaches and require about two weeks for maturation. Most points seem logically satisfactory but a few remain ambiguous.

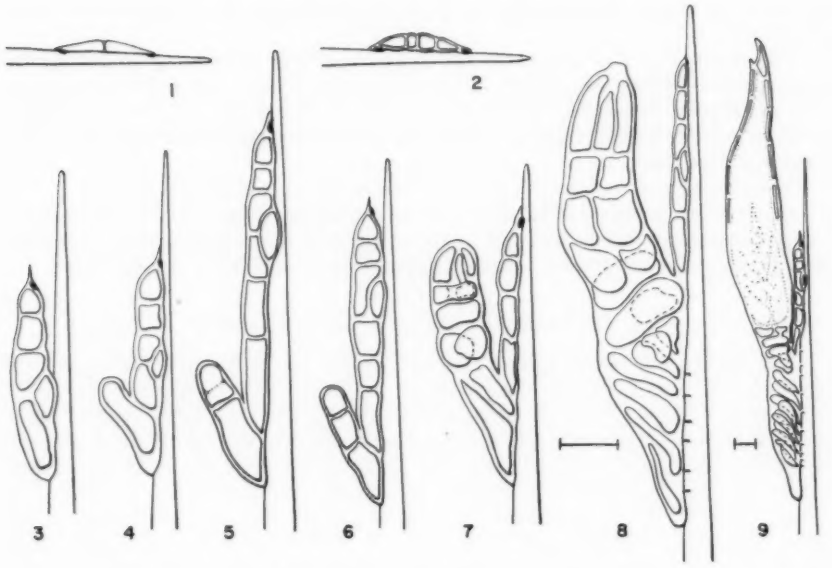
The structure of haustoria is omitted (see Richards and Smith, 1955b).

All drawings were made to scale free-hand using an ocular grid and ruled paper. Certain stages have been illustrated by photographs in preceding parts of this series; such photographs are referred to at the appropriate places in the present paper.

Development of the Female Plant

The spores are released from the asci within the perithecia (photographs in Richards and Smith, 1955a). At the time of ejection and transfer they are in the 2-cell stage (Fig. 1) but soon become 4-celled. In size they range from 2–3 μ in width and 20–30 μ in length. In the 2-cell stage the two cells are of about the same size and make a symmetrical spore, but in the 4-cell stage the cells may appear similar or quite dissimilar with no particular significance evident for the differences (Figs. 10–14). Many spores do not develop beyond this stage, or at least do not do so promptly (Richards and Smith, 1955a). While spores occasionally develop on other parts of the body, almost all of the mature plants of *H. stylopygae* are found on the antennae of the host. We did not find any way to distinguish spores that would develop into male plants from those that would develop into female plants until differentiation subsequent to the 4-cell stage.

On setae the first evidence of further growth is a considerable increase in volume, although this increase does not seem to be a constant amount and is not necessarily as great as the differences between Figures 2 and 3. Beyond this initial change in volume, the first two cells and the fourth cell do not change further. Up to this stage there has been no apparent difference between the two ends of the spore, but with growth proceeding basally the two ends become obviously different (Fig. 3). The base of the plant is always towards the base of the seta, but it is not known whether this is due to orientation being produced by orientation on the host or whether only those spores so oriented develop (see Richards and Smith, 1955a). The third or subbasal cell grows basally over the fourth or basal cell, and divides repeatedly to give a row of three to five cells. The terminal cell bulges away from the seta (Fig. 4) and divides to give two cells (Fig. 5). The apical one of these promptly divides to give a third cell (Fig. 6). At this stage, then, we have a row of five to seven cells which will not develop further (the primary receptacle), and a basal obliquely protruding group of three cells which will form the remainder of the plant. The basal of these three cells divides repeatedly extending the plant down the shaft of the seta (Figs. 7–9) to form the secondary receptacle, processes from individual cells penetrating as minute haustoria into the shaft of the seta (photographs in Richards and Smith, 1955b). The middle one of the row of three cells divides to give the basal cells of the perithecium, and the apical one of the three cells divides first transversely, then longitudinally to develop into the peri-



FIGURES 1–9. Development of female plant of *H. stylopygae* growing on antennal setae of the oriental cockroach. Figures 1–8 at same magnification, Figure 9 smaller, the bars representing 10 μ. See text for explanation.

thecium (see below). This cell lineage is diagrammed as the top sequence in Figure 55.

When growing on setae, the characteristic structure shown in Figure 9 is produced. Only the perithecium resembles that of plants growing on the general surface of the antenna. The secondary receptacle is not only of grossly different shape but its component cells are also different, and there is no trace of development of the peculiar "shield." In some cases, a spore germinating near the base of a seta will reach the base and spread across the antennal surface (this is common for the species *H. ectobiae*); in these cases the plant changes more or less from the growth pattern on setae to that on the general body wall of the antenna.

Female plants growing on setae are usually alone and usually have only a single perithecium. Sometimes one finds both a male and a female plant growing together on a seta. And, occasionally, a pair of female plants are found alongside one another on a single seta—it is not known whether these arise from one spore or two.

On the general antennal surface (*i.e.*, not on setae) the pattern of development of female plants is more complex (Figs. 15–36). Development begins as on setae by swelling of the 4-cell stage and growth of the third (subbasal) cells in the basal direction (Fig. 15). The prolongation of the third cell becomes constricted off (Figs. 17, 18) and immediately develops as a protrusion which divides to give two cells, the more apical one soon dividing to give two, then three, cells (Fig. 20). The

subsequent history of this protruding row of cells is similar to that of the protruding basal row of Figure 6. The apical one eventually gives rise to the perithecium, the middle one gives rise to the basal cells of the perithecium, and the basal one divides longitudinally (Figs. 21–24) to give rise to a basal row of five to six elongated cells (Fig. 25) which forms the secondary receptacle (and sends a large haustorium penetrating through the insect's cuticle into the underlying epidermis; see Richards and Smith, 1955b).

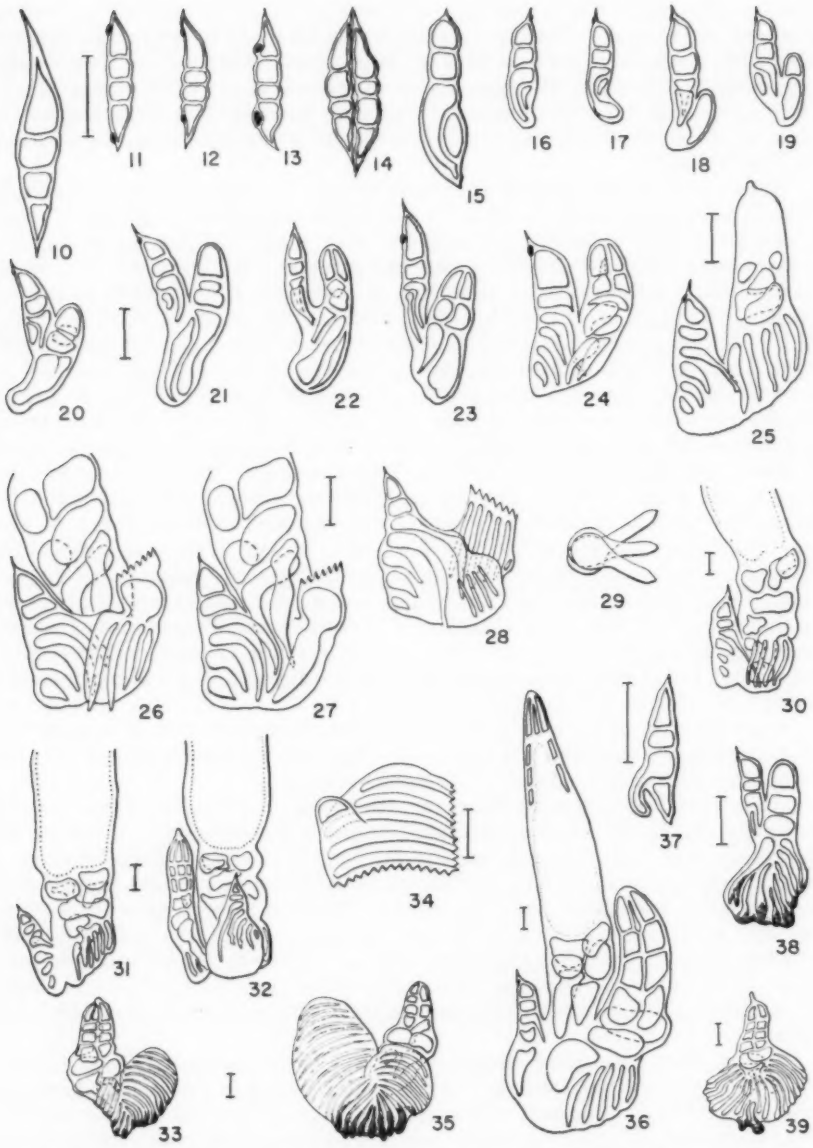
But while the above development is taking place, the original group of four cells (Figs. 16–23) increases to six (Fig. 24) or occasionally seven (Figs. 28, 32), and comes to constitute a distinct portion of the plant (called the primary receptacle). It is uncertain which cells divide to give this increase. In Figures 15–17 it seems that the fourth cell is being by-passed (as on setae) but later it seems to develop (Figs. 18, 22) and to form a projection similar to that from the third cell (Fig. 23). With the increase in cells of the primary receptacle to six, it is found that the third, fourth and fifth all have long processes (Figs. 26–28). These processes do not lie in the same plane but diverge in the manner diagrammed in Figure 29 (which is reconstructed by focal plane analysis of the section drawn in Figure 28; it represents Figure 28 as it would appear if viewed along the plane of the page). Each of these processes buds off a cell which divides and gives rise to a secondary receptacle and a perithecium. By this stage the structure is too complicated for satisfactory analysis from whole mounts. Sections such as those drawn in Figures 26–28 show the process from the third cell extending to the base of the oldest perithecium, the process from the fourth cell extending to the base of a second and much younger perithecium (as Figs. 32 and 36), and the process from the fifth cell extending to a still younger perithecium. (Figures 26 and 27 are drawn from the same section, the base of the secondary receptacle of the younger perithecium being omitted from Figure 27 to permit showing underlying cells.) This cell lineage is diagrammed as the middle sequence in Figure 55.

After budding off of cells to form secondary receptacles and their perithecia (two secondary receptacles shown in Fig. 28), the primary receptacle remains static but can be found underneath the mature plant.

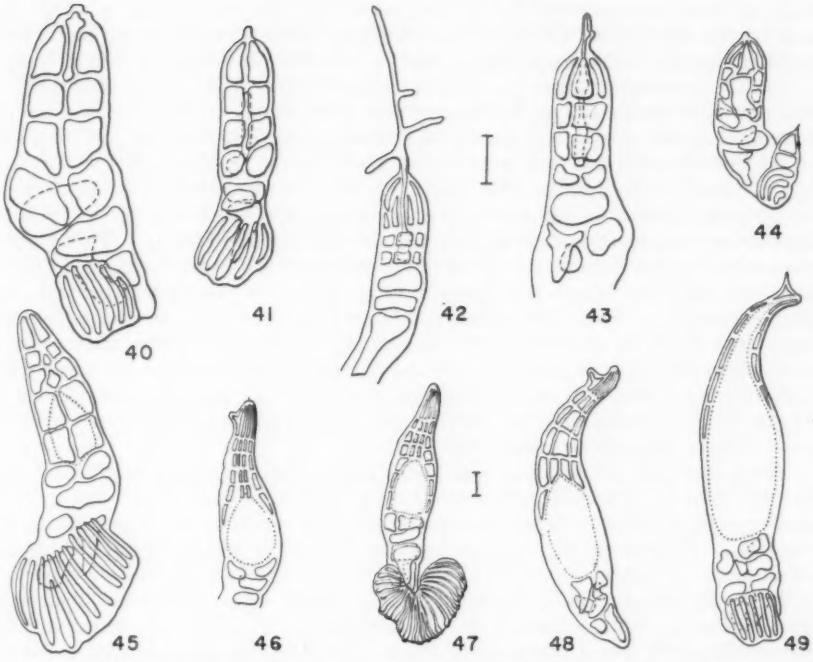
After several perithecia are underway, the secondary receptacle of one of them, seemingly that of the oldest perithecium, begins to hypertrophy. A lateral outgrowth develops involving an increase in the number of elongated cells of the secondary receptacle and a turning of these new cells so that they extend transverse to the plant's axis rather than parallel to it (Fig. 33). Or such growths may develop on both sides (Figs. 35, 47). This growth gives rise to the peculiar "shield" covering the base of the plant (but found only in *H. stylopygae* and *H. periplanetae*). Outgrowths from both sides can give rise to paired "shields" (Fig. 35). Sometimes the developing "shields" themselves grow little additional lobes (Fig. 34) but such are not common.

Shortly after the "shield" begins to grow it begins to blacken basally in *H. stylopygae* (not in *H. periplanetae*) as shown by stippling in Figure 33. Later the basal blackening becomes more extensive (Fig. 35). This darkening is a chemical process showing histochemical reactions similar to those of the host's cuticle during sclerotization (Richards, 1954).

Occasional plants are found which do not fit into the above sequence. Sometimes germinating spores develop in the manner shown in Figure 37; this type of



FIGURES 10–39. Development of female plant of *H. stylopygae* growing on general surface (not on setae) of antennae of the oriental cockroach, with particular reference to the basal receptacles and "shield." See text for explanation. Drawn with primary receptacle to the



FIGURES 40–49. Development of the perithecium of female plants of *H. stylopygae* from stages equivalent to Figures 8 and 25 to maturity. See text for explanation. Magnifications: vertical bars 10 μ (Figs. 40–45 at same magnification, Figs. 46–49 at same).

outgrowth is the common expression of incipient germination on greased collodion membranes but is rare on antennae. We have no idea whether or not such spores can continue development on antennae. Occasional plants show increase in number of elongated cells of the secondary receptacle, either irregularly (Fig. 38) or regularly (Fig. 45) or symmetrically (Fig. 39). Too few such cases were seen for us to have any idea of the subsequent development of such plants.

After their initiation, the development of perithecia is similar for plants growing on setae and on the general antennal surface. The apical cell cut off by the first division of the basal cell of Figures 4 and 18 divides transversely. The row of three cells formed (Figs. 6, 20) gives rise, respectively, to the secondary receptacle (plus "shield"), the basal cells below the perithecium, and the perithecium, as already mentioned. The middle one of the original row of three divides more or less irregularly to give rise to a group of relatively large cells of various shapes (Figs. 26, 30, 40–44). These basal cells of the perithecium are multinucleate, and some

left, secondary receptacle and perithecia to the right except for Figure 32 where the primary receptacle is uppermost (young perithecium on left) and Figures 33, 35 and 39 which do not show a primary receptacle. Magnifications: all vertical bars represent 10 μ and refer to adjacent sketches (Figs. 10–15 at same magnification, Figs. 16–29 at same).

or all of them are interconnected by cytoplasmic strands and bridges (photographs in Richards and Smith, 1955b). The visible cytoplasmic connections are limited to this group of cells; they do not extend on into the secondary receptacle basally or into the perithecium distally. The apical cell of the original row of three first divides longitudinally (Figs. 22–24) and then transversely to give rise to a row of three or four sets of four cells each (Figs. 8, 40). At first these are not distinctly set off from the cells that will form the basal cells of the perithecium, but with further divisions giving smaller cells (Figs. 42, 43) the distinction becomes obvious. At the stage shown in Figure 41, one of the basal cells of this group becomes displaced to the center and develops a process which grows distally. This process reaches and grows through the tip of the perithecium to form an elongated (up to 43 μ) and variously branched trichogyne (Fig. 42). No two trichogynes have the same appearance. Soon the trichogynes become more or less covered with spermatia (not shown in drawing) from male plants. With fertilization accomplished, the trichogynes begin to regress while the portion in the center of the developing perithecium enlarges to form a distinct ascogonium. The ascogonium is shown in Figure 43 by a dotted line, but its size and distinctness are more clearly seen in serial sections through the plant (Fig. 44).

After fertilization and regression of the trichogyne, the young perithecium begins to elongate (Fig. 45) and there is seen a differentiation of several rows of smaller cells apically contrasting with larger cells surrounding the swelling ascogonium. Subsequent divisions increase the number of smaller apical cells as the perithecium continues to elongate (Figs. 46–49); these cells form the apical portion of the perithecium including the canal through which spores later make their exit. Simultaneously, the ascogonium swells enormously (Figs. 46–49) stretching the surrounding cells into a thin surface sheet. Details of ascogenesis within the ascogonium were not worked out because of the poor visibility in whole mounts and the lack of a sufficient number of early and intermediate stages in our serial sections. Clearly, however, by the stages shown in Figures 9 and 49, *i.e.*, by the time the perithecium has developed fully the characteristics used by taxonomists for identification of species, asci have developed.

The first perithecium developed on multiperithecial plants reaches maturity (presumably including functional maturity) while the second and third perithecia are still quite young (Figs. 32, 36). Later all the perithecia become mature, and the infected antennae show the customary appearance of plants with perithecia all of which are mature (photographs in Richards and Smith, 1955a, 1955b). One has to have infections including relatively young plants to obtain specimens with perithecia of obviously different ages.

Development of the Male Plant

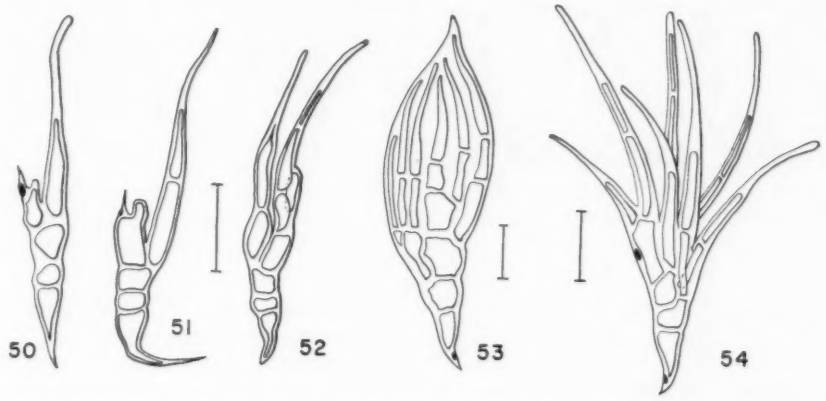
Male plants of *H. stylopygae* seem to be considerably less common than female. In contrast to the female plant, the male is very simple. It also appears to develop in the same manner whether on setae or on the general antennal surface (but is relatively seldom seen on setae in this species).

The 4-cell spore of the male plant attaches to the insect's cuticle by the basal cell (with a small haustorium penetrating therefrom; see Richards and Smith, 1955b), and the apical cell begins to bud off antheridia (Fig. 50). These appear

to be budded off one at a time (Fig. 51) until a considerable tuft has developed (Figs. 53, 54). The mature male plant, then, consists of a stalk formed from the basal three cells and a tuft of antheridia budded off the apical cell. The cell lineage is diagrammed as the bottom sequence in Figure 55.

The antheridia initially consist of a single elongated cell (Fig. 50) but this soon divides to give a linear array of two or three cells with a tip and duct extending farther (Figs. 51–54). The spermatia produced (not drawn) are small blobs less than a micron in each dimension (seem to average about $0.5 \times 0.8\,\mu$).

In some cases the tuft of developing antheridia is encased in a sheath (Fig. 53). In other cases no such sheath is evident. At maturity the tuft presents a picture similar to Figure 54 or with a few more antheridia.



FIGURES 50–54. Development of male plant of *H. stylopygae* on antennae of the oriental cockroach. See text for explanation. Magnifications: vertical bars 10 μ.

DISCUSSION

Despite the limitations imposed by the method of seriating developmental stages found in different specimens, it has been possible to work out a logical sequence for most of the ontogenetic details. Of most general interest is the difference in cell lineage for male and female plants, and for female plants growing on setae in contrast to ones growing on the general antennal surface. These differences are summarized in Figure 55 where "A" and "B" represent the cells of the 2-cell spore giving rise respectively to cells "A_1," "A_2" and "B_1," "B_2" of the 4-cell spore.

In the female plant growing on setae, the 4-cell spore develops into a row of six or seven cells by repeated division of the third or subbasal cell (B_1). The most basal one of this row (B_{1b} or B_{1c} depending on whether 6 or 7 cells) differentiates into a cell (C) which divides to give basally a cell (D) which produces a secondary receptacle (SR) and haustorium (H), and obliquely apically a cell (E) which produces a perithecium (P). Cells "A_1" to the next to the last of the "B" series remain as the so-called primary receptacle. No "shield" is developed.

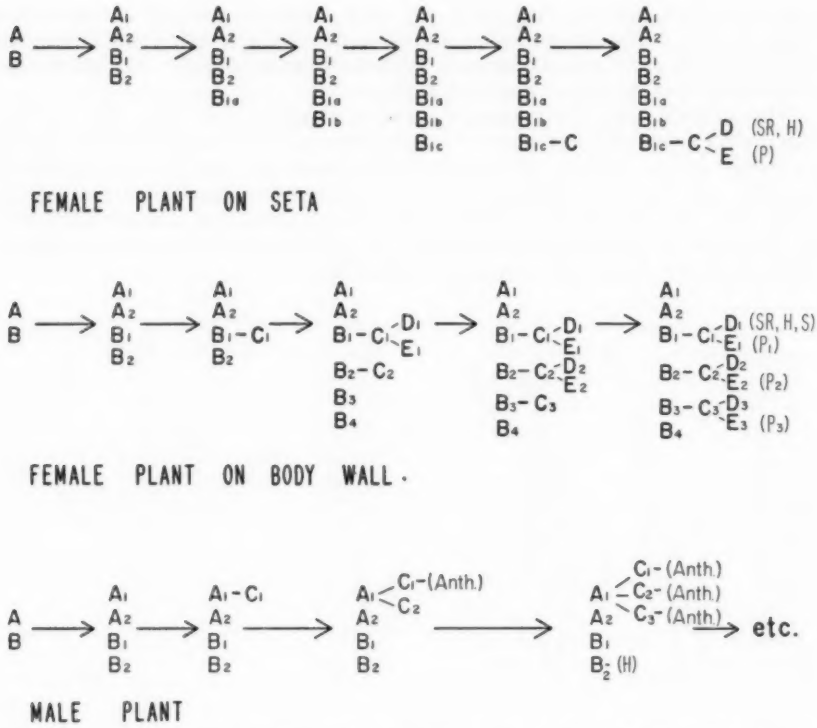


FIGURE 55. Diagrams of cell lineage in developing plants of *H. stylopygae*. See text for explanation.

In the female plant growing on the body wall proper of the antenna, the third or subbasal cell (B_1) produces by budding a cell (C_1) which in turn gives rise to two cells (D_1 and E_1) which produce the secondary receptacle (SR) and perithecium (P), respectively. But, simultaneously some of the "B" cell series have divided to give additional cells "B_3" and "B_4" (and sometimes a "B_5"). Cell "B_2" then differentiates in a manner similar to that of "B_1," and later cell "B_3" does likewise. When seven cells are present instead of six it seems that cell "B_4" also does likewise at a still later time. Cells "A_1," "A_2," and the last one of the "B" series contribute nothing except that the whole of the "A" and "B" series, after budding off of the "C" series, remain as the so-called primary receptacle. And, finally, the first secondary receptacle (SR) gives rise to the single large haustorium (H) and then later hypertrophies to produce the peculiar "shield" (S) covering the base of the plant.

In the male plant, the basal cell (B_2) forms the attachment to the host and sends a haustorium (H) into the insect, while the apical cell (A_1) buds off cells (the "C" series) each of which gives rise to an antheridium (Anth.).

Clearly, in the female plant the "A" series contributes nothing (except for remaining as part of the primary receptacle) and the whole visible plant develops from the "B" series, perhaps just from the subbasal cell (B_1), whereas in the male plant attachment is by the basal cell (B_2) and antheridia develop from the apical cell (A_1). Cell lineage is, then, fundamentally different in the two sexes.

The two types of growth of female plants seem to us merely products of growth restriction. In both types growth is initiated by the subbasal cell (B_1). Presumably growth on the general surface of the antenna is unrestricted. Several of the cells of the "B" series then bud off one after another processes which give rise to secondary receptacles and perithecia. The oldest of the secondary receptacles later hypertrophies to give the peculiar growth called the "shield." On a seta the secondary receptacle either has to protrude laterally into space or grow along the seta —it grows along the setal shaft. Rationalizing, once the terminal cell has begun to form a receptacle and perithecium, there simply is no place for another cell of the "B" series to develop unless it moved around the seta (which it at least usually does not do although some paired plants seen on setae could be interpreted as having arisen in this manner). On both body wall and setae the secondary receptacles hypertrophy later in development; on the body wall this growth gives the "shield," on setae it gives an elongation of the secondary receptacle with increasing cell number. It seems to us that female plants of *H. stylopygae* develop on an essentially similar pattern in both cases but show considerable plasticity to fit the geometric limitations of the particular surface on which they are growing.

Summary

The development of male and female plants of the dioecious fungus *Herpomyces stylopygae* on cockroach antennae is described. Cell lineage is fundamentally different for male and female plants. The gross structure and apparent cell lineage are different for female plants growing on setae in contrast to ones on the body wall. An interpretation in terms of developmental plasticity and geometric limitations of the host's surface is suggested for the two types of growth of female plants.

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DEVELOPMENTAL MODIFICATIONS IN THE SAND DOLLAR CAUSED BY ZINC CHLORIDE AND PREVENTED BY GLUTATHIONE[1]

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Problems dealing with growth and differentiation are of obvious importance. The modification and control of cleavage rate, developmental patterns and differentiation in echinoderms by the use of chemical agents has greatly interested the writer for several years. In 1947, work was begun which involved testing the effects of various enzyme inhibitors and activators on cleavage and developmental patterns of the early larva. Many agents, which reputedly affect one or more enzymes or enzyme systems, have been used. As the work has progressed the problem has become increasingly more complex. At the present moment it appears that the term "specific" as applied to morphological changes from enzyme inhibition or activation is a relative term. Granted that a particular agent may affect a certain enzyme specifically, the effect on developmental pattern may be quite similar to the effect produced by another agent on another enzyme, provided both enzymes are in the complexity of interactions leading to a particular modification of structure. Most enzyme inhibitors affect more than one enzyme. Many have multiple effects. Some activate one enzyme while inhibiting another. Concentrations of inhibitors are extremely important. Some inhibitors affect many enzymes in high concentrations but in lower concentrations only certain of the enzymes.

Despite the extreme complexity of the problem certain effects of enzyme inhibitors on development have become increasingly clear. Totally different agents may cause the same modification in developmental pattern but modifications may differ widely between various groups of inhibitors. One group of agents may be particularly effective in causing ectodermization while another may cause exogastrulation and entodermization almost 100 per cent of the time if concentrations and exposure periods are controlled. Some agents have little or no effect on the gastrulation process but will cause the embryo to develop around a radial symmetry. Some agents affect the differentiation of skeletal spicules more than other agents. It has recently been found (Rulon, 1952, 1953a, 1953b) that selenium, cobalt, and nickel are all effective in causing the development of radial embryos which have elongated in the polar direction and differentiated with respect to a new pattern. At present our knowledge is extremely limited on many of the chemical, physical, and biological factors in development. This author is making an attempt to correlate certain morphological changes with chemical action. He believes that with data from a large number of experiments involving the use of enzyme inhibitors and activators,

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and with morphological changes as markers, it should be possible to determine certain common denominators between developmental changes, enzyme activity, enzymogenesis, histogenesis, etc.

The present report deals chiefly with experiments in which only one agent was used. Zinc chloride was chosen as the test agent because of its known action on certain enzymes. Histidase is inactivated while prolidase, carnosinase, certain dipeptidases, alkaline phosphatase, and pyrophosphatase I are activated by the zinc ion (see Sumner and Somers, 1953, for references). Zinc is also known to react with sulfhydryl groups (Potter and DuBois, 1943) and it is known that the sulfhydryl radical is essential to the activity of many, but not all, enzymes (Sumner and Somers, 1953).

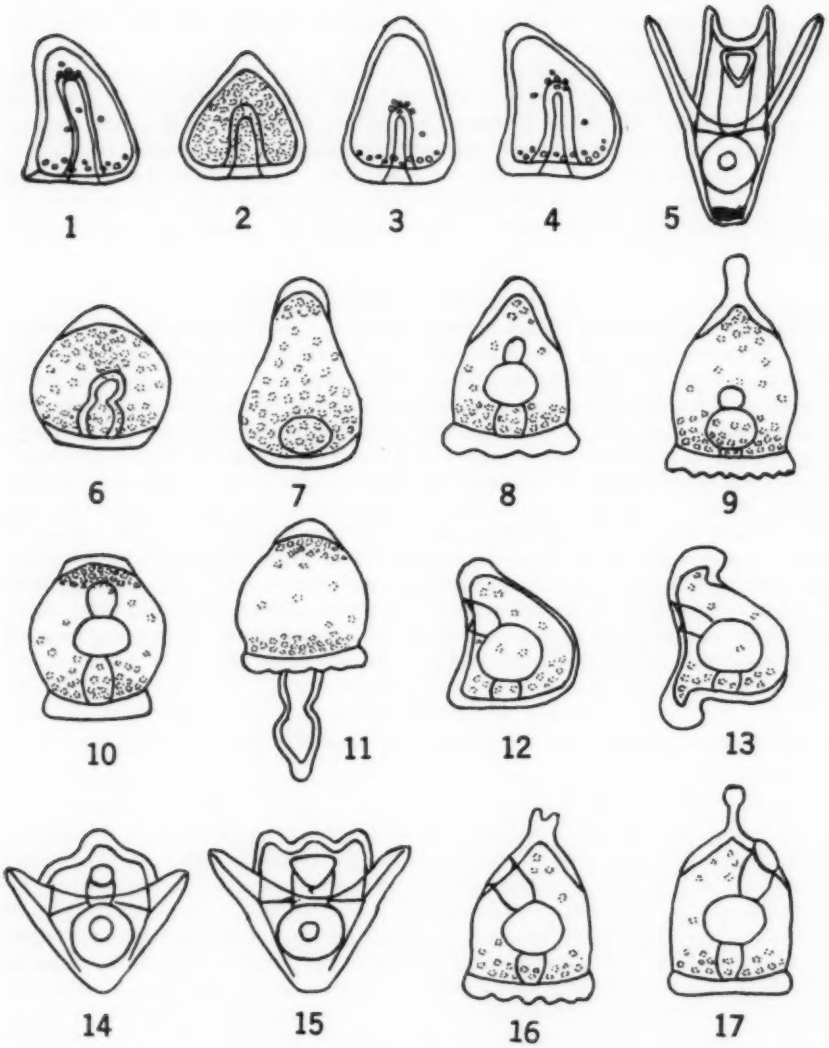
Material and Methods

The adult sand dollars (*Dendraster excentricus*) used in this study were dredged from Monterey Bay near the Hopkins Marine Station, Pacific Grove, California, during the summers of 1953–54. They were maintained in running sea water in the laboratory. Ripe eggs were removed from mature females by cutting away the oral surface of the test and allowing them to exude from the ovaries. A medicine dropper was used to transfer the eggs to sea water where they were washed several times. Fertilization was accomplished by adding a few drops of sperm suspension. All experiments were performed in the aquarium room of the Station where the temperature varied by no more than one degree from 18 degrees C. All eggs were kept in uncrowded lots in finger bowls and out of direct sunlight. Smoking was not permitted in the room. In preliminary experiments, lots of eggs were placed in sea water solutions of zinc chloride ranging from $M/100$ to $M/320{,}000$. Since the higher concentrations were very toxic only experiments using concentrations ranging from $M/10{,}000$ to $M/320{,}000$ are reported here.

Experimental

1. *Continuous exposure to zinc chloride beginning with the one-cell stage*

When newly fertilized eggs were placed directly into $M/10{,}000$–$M/20{,}000$ solutions of zinc chloride the rate of cleavage was slightly inhibited as noted by observations of the six-hour blastula. No such inhibition was seen in $M/40{,}000$–$M/320{,}000$ solutions. After 24 hours, when the controls were normal preplutei (Fig. 1), the larvae in $M/10{,}000$ were chiefly slow-moving, cell-filled, radial, inhibited gastrulae. Approximately 50 per cent were stunted, conical forms (Fig. 2) and a few showed slight bilaterality. In $M/20{,}000$, the larvae were almost all conical gastrulae with fewer internal cells. Roughly 50 per cent were free-swimming and there was an occasional exogastrula. Those in $M/40{,}000$ were chiefly free-swimming conical types with about 25 per cent exogastrulation. Most of the larvae in $M/80{,}000$ were slightly elongated, radial, conical gastrulae (Fig. 3) with basal regions slightly thicker than those of larvae developing in higher concentrations. An occasional bilateral form had developed in this solution and approximately 25 per cent had undergone exogastrulation. At 24 hours, $M/160{,}000$, gave about 75 per cent slightly bilateral gastrulae and 25 per cent exogastrulae. All the embryos in $M/320{,}000$ were late bilateral gastrulae (Fig. 4) that were not far behind the controls.



FIGURES 1–17. Figure 1, normal 24-hour prepluteus. Figures 2–4, 24-hour larvae exposed since fertilization to $M/10{,}000$ (Fig. 2), $M/80{,}000$ (Fig. 3) and $M/320{,}000$ (Fig. 4) solutions of zinc chloride. Figure 5, normal 48-hour pluteus. Figures 6–15, 48-hour larvae exposed since fertilization to $M/10{,}000$ (Figs. 6–7), $M/20{,}000$ (Fig. 8), $M/40{,}000$ (Figs. 9–11), $M/160{,}000$ (Figs. 12–14), and $M/320{,}000$ (Fig. 15) solutions of zinc chloride. Figures 16–17, 48-hour larvae exposed since the 6-hour blastula stage to $M/80{,}000$ zinc chloride.

After 48 hours, when the controls were well-developed, bilateral plutei (Fig. 5), the larvae in *M*/10,000 were radial, cell-filled, slow-moving gastrulae (Figs. 6, 7) with many slightly elongated in the polar direction. In *M*/20,000 the picture differed slightly in that the apical thickening was usually more pronounced, the enteron was better developed, and the basal region was commonly thickened into a fluted, ciliated band (Fig. 8). There was approximately 10 per cent exogastrulation. In *M*/40,000, all had radial symmetry, basal ciliated bands, and apical thickenings which often extended upward as buds of ectoderm (Figs. 9, 10). Here, also, approximately 10 per cent showed exogastrulation (Fig. 11). In *M*/80,000 the situation was not much different but in *M*/160,000 most of the larvae were bilateral, without arms, and with little or no skeleton (Fig. 12). Commonly a ventral ciliated band was over-developed (Fig. 13). There were a few radials but some had developed into recognizable plutei (Fig. 14). In *M*/320,000 about 50 per cent were still bilateral forms without arms or skeleton while the remaining 50 per cent graded up to wide-angled plutei with wide, but poorly differentiated, oral lobes (Fig. 15).

These experiments, dealing with the continuous exposure of newly fertilized eggs to various concentrations of zinc chloride, show that high concentrations inhibit cleavage but that lower concentrations, which have no noticeable effect on cell division, do cause marked changes in developmental pattern. The chief effect of strong solutions is in causing the development of radial, poorly-differentiated larvae. As the strength of the solution was decreased, thickened ciliated basal bands appeared and the larvae elongated in the polar direction. In the weaker solutions bilaterality developed and in the weakest, plutei with increased ventral areas but with poorly differentiated oral lobes were found. A slight bilaterality, that was present at 24 hours in M/10,000, had disappeared by 48 hours. Lots showing 25 per cent exogastrulation at 24 hours showed only 10 per cent of such development at 48 hours. Skeleton was completely inhibited in all concentrations above *M*/80,000 and in solutions as low as *M*/320,000 only 50 per cent of the larvae showed any skeletal development.

2. *Continuous exposure to zinc chloride beginning with the mid-blastula stage*

When 6-hour blastulae were placed in *M*/10,000 zinc chloride solution all had developed, after 18 hours, into radial, conical forms (as in Figs. 2, 3). Most were free-swimming, a few had an excess of internal cells and there was only an occasional exogastrula. In *M*/20,000 and *M*/40,000 radial, conical forms had developed but in the case of the lower concentration the gut was usually better differentiated and commonly bent toward one side. In *M*/80,000 most were still free-swimming conical gastrulae but slight bilaterality could be seen in a few. It was also noted that many were irregular around their basal regions as if fluted circles of cilia were developing. Approximately 10 per cent of the larvae had undergone exogastrulation in this solution. In *M*/160,000 and *M*/320,000 the larvae had become bilateral (as in Fig. 4) and those in the weakest solution showed a trace of skeleton.

At 48 hours the larvae, that had been in *M*/10,000 zinc chloride since the 6-hour blastula stage, were not very different from those that had been in this concentration since fertilization. That is, they were slow-moving, radial forms with an excess of internal cells (as Figs. 6, 7). In *M*/20,000 and *M*/40,000 the blastulae had

developed in a manner similar to those exposed from the one-cell stage to comparable solutions except that with the blastulae, very few exogastrulae appeared. The situation in *M*/80,000 was not far different except that here a well-developed enteron was the rule and commonly a stomodaeum developed (Figs. 16, 17). In *M*/160,000, at least 50 per cent showed bilaterality and graded into plutei possessing short anal arms with skeletal spicules. The remainder resembled those from higher concentrations. With *M*/320,000, practically all were inhibited, bilateral plutei with short anal arms at various angles, asymmetrical, or askew and a few with widened, but poorly differentiated, oral lobes.

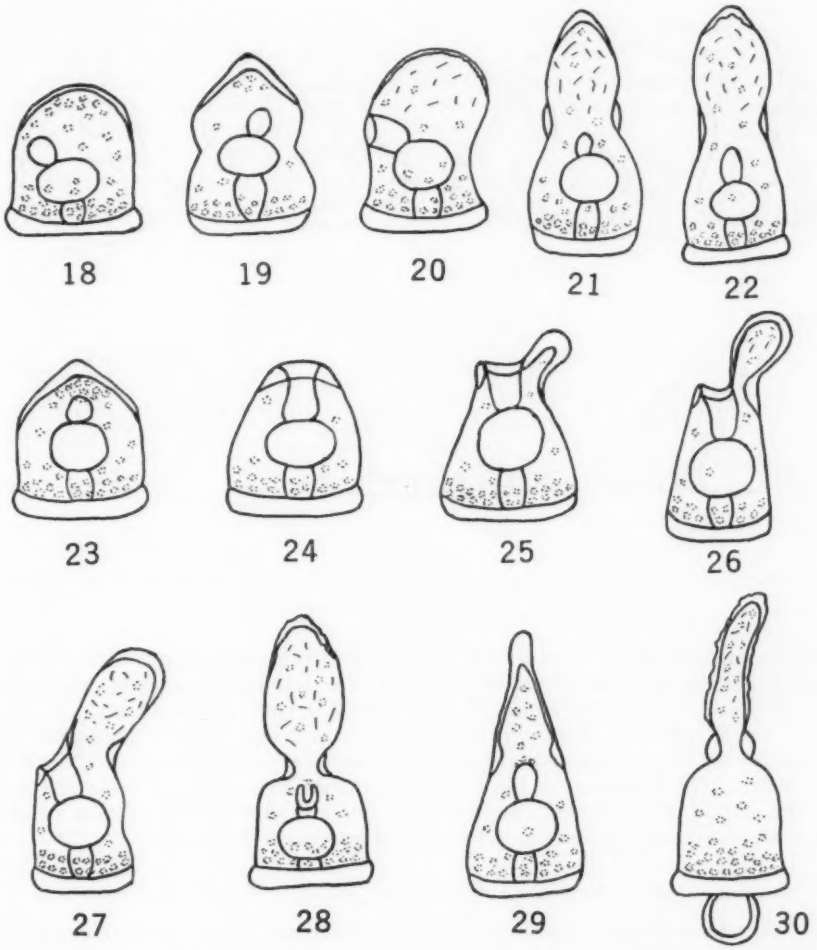
The data from the above experiments show that treatment with zinc chloride after cleavage gives larvae that are only slightly less modified than those exposed continuously after fertilization. The chief differences were that fewer exogastrulae developed, skeleton differentiated in slightly higher concentrations, and stomodaea appeared in solutions that had previously been inhibitory.

3. Exposure of newly fertilized eggs to zinc chloride for six and twenty-four hours

Eggs treated with *M*/10,000–*M*/40,000 zinc chloride solutions for only the first six hours developed into inhibited, bilateral plutei when returned to sea water. At 48 hours these embryos possessed short anal arms at aberrant angles, poorly differentiated oral lobes, and 10 per cent exogastrulation. Such inhibition was more noticeable in the higher concentrations. Similar treatment with *M*/80,000–*M*/320,000 showed little or no effects of the agent.

When newly fertilized eggs were treated for the first 24 hours with *M*/10,000–*M*/20,000 zinc chloride and then returned to sea water they developed chiefly (by 48 hours) into radial forms with differentiated entera and basal rings of cilia (Figs. 18–22). At least 50 per cent of these embryos showed considerable polar elongation (Figs. 21, 22). In the weaker solution, polar elongation was more pronounced and stomodaea were commonly present. With 24 hours exposure to intermediate concentrations (*M*/40,000–*M*/80,000) all of the larvae developed radial symmetry, well-differentiated entera (with 10 per cent exogastrulation) but no skeleton (Figs. 23–30). Approximately 50 per cent showed apical lobes or extensions (Figs. 28–30) which were often quite large. Up to 50 per cent showed the mouth at the extreme apical end (Fig. 24) while many showed the mouth at one side of an apical lobe (Figs. 25–27).

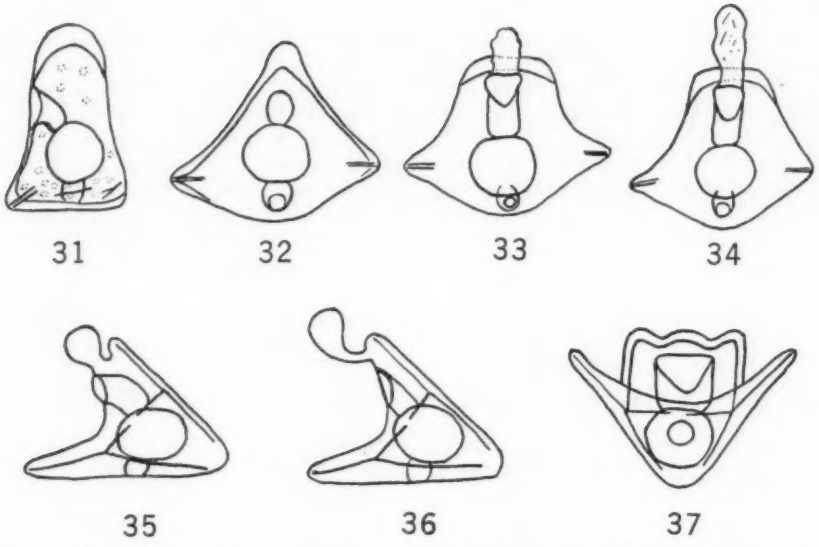
Following 24 hours treatment with low concentrations (*M*/160,000–*M*/320,000) the eggs developed chiefly into bilateral plutei showing all degrees of inhibition (Figs. 31–37). Anal arms were short and at increased angles. Modifications were more pronounced in the stronger of these two solutions (Figs. 31–34) where a few approached the elongated radial forms (Fig. 31). Following exposure to the weaker solution (*M*/320,000) a few developed into plutei that were not far from the controls (Fig. 5) except for broad, poorly-differentiated oral lobes and slightly shortened anal arms at increased angles (Fig. 37). All developed some skeleton. One of the most interesting modifications found in these forms was the appearance of a thin-walled sac in the region of the upper lip of the stomodaeum (Figs. 33–36). This sac was undoubtedly a small apical lobe, comparable in origin to the apical lobes of more modified embryos (Figs. 25–27) and arising from a



FIGURES 18–30. Figures 18–22, 48-hour larvae treated for the first 24 hours with $M/10{,}000$–$M/20{,}000$ zinc chloride and then returned to sea water. Figures 23–30, 48-hour larvae treated for the first 24 hours with $M/40{,}000$–$M/80{,}000$ zinc chloride and then returned to sea water.

region that had escaped the controlling factors normally found in the differentiation of an oral lobe.

In general, these experiments have demonstrated that high concentrations of zinc chloride give over-all inhibition if administered only during cleavage (first six hours) while low concentrations have little or no effect if used only during cleavage. It was also shown that the removal of larvae from zinc chloride solutions after 24 hours permits a greater degree of differentiation than when the ex-



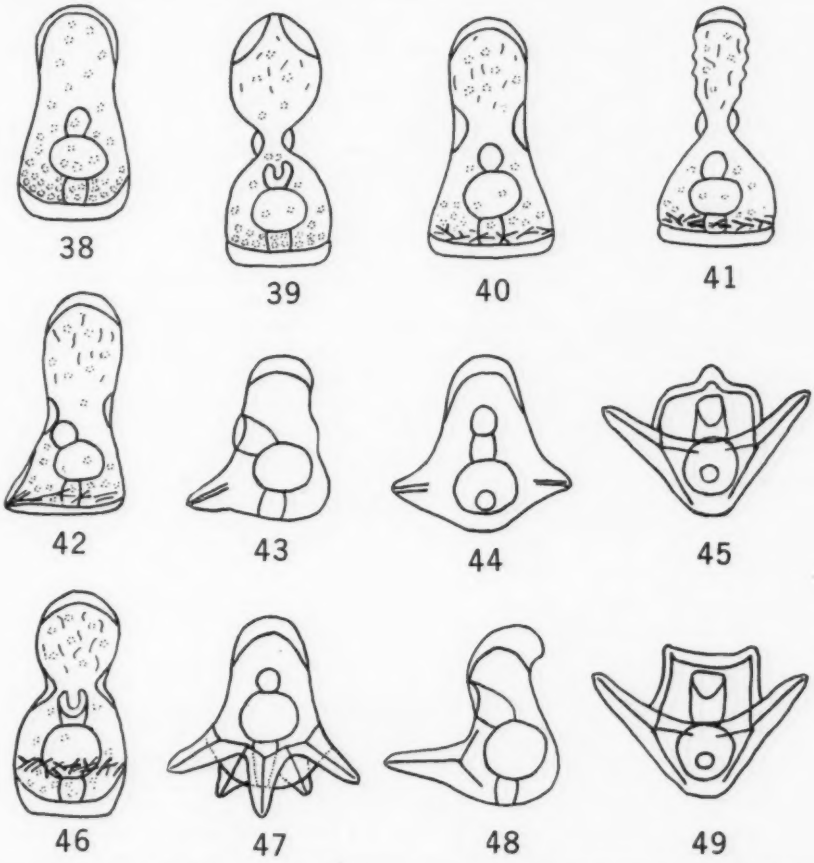
FIGURES 31–37. 48-hour larvae treated for the first 24 hours with $M/160{,}000$–$M/320{,}000$ zinc chloride and then returned to sea water.

posure was continuous. In high ($M/10{,}000$–$M/20{,}000$) and intermediate ($M/40{,}000$–$M/80{,}000$) concentrations for 24 hours there was radial symmetry and polar elongation but with apical and basal lobes developing and differentiating. Stomodaea also appeared in these experiments which may indicate that the factors responsible for such differentiation are not very sensitive to inhibition during the first 24 hours. The appearance of the stomodaeum at the apical end may indicate a part of the phenomenon of ventralization in which the ventral area extends more rapidly than the dorsal and in this case pushes the stomodeal region apically.

4. Exposure of mid-blastulae to zinc chloride for six and eighteen hours

Whereas six hours exposure to high concentrations of zinc chloride, beginning immediately after fertilization, gave only over-all inhibition and lower concentrations had little or no effect the situation was quite different when the 6-hour blastula was exposed for a similar period. Six hours exposure to $M/10{,}000$–$M/20{,}000$ gave 75–90 per cent radial elongated forms (Figs. 38–41) with thin apical regions or lobes. The remainder graded into inhibited bilateral plutei (Figs. 42–45). Some of these were radial and enlarged apically but bilateral basally (Figs. 42, 43). In $M/20{,}000$, most of the bilaterals had skeleton and most of the elongated radials had rings of skeletal spicules around their bases (Figs. 40, 41). No exogastrulae were found. Approximately 60 per cent of the blastulae exposed for six hours to $M/40{,}000$ developed into bilateral forms (as Figs. 42–45), commonly elongated in the polar direction (Figs. 42–44) but with short anal arms. The remaining 40 per

cent were radial and usually elongated. All of these had circles of skeletal spicules around their bases and many formed circles of short arms (Figs. 46, 47). Following treatment with $M/80{,}000$ most of the larvae developed bilaterality and two anal arms at increased angles (Figs. 48, 49). The oral lobes were commonly extended and poorly differentiated (Fig. 48). There were many irregularities and an occasional exogastrula. Treatment with $M/160{,}000$–$M/320{,}000$ gave chiefly normal plutei although there were still a few minor modifications to be found in the stronger of these two solutions.



FIGURES 38–49. Figures 38–41, 48-hour larvae exposed for six hours to $M/10{,}000$–$M/20{,}000$ zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water. Figures 42–47, 48-hour larvae exposed for six hours to $M/40{,}000$ zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water. Figures 48–49, 48-hour larvae exposed for 6 hours to $M/80{,}000$ zinc chloride beginning at the 6-hour blastula stage and after treatment returned to sea water.

The exposure of blastulae to the various concentrations of zinc chloride for 18 hours, and followed by return to sea water, gave forms that were very similar to those from exposure to the same solutions for 24 hours beginning shortly after fertilization. Skeleton and slight bilaterality was found in a few at *M*/80,000. Exogastrulae occurred only occasionally but stomodaea opened at the apical end (Fig. 24) in as high as 50 per cent following treatment with *M*/40,000–*M*/80,000.

These experiments show that six hours exposure to zinc chloride after cleavage is highly effective in causing the development of radial symmetry and polar elongation. This is to be contrasted with exposure during the six-hour period of cleavage which is relatively ineffective. Skeleton was not greatly inhibited during the second six hours of treatment and commonly differentiated with reference to a radial symmetry even to the extent of appearing in a circle of radial arms. That treatment during the cleavage period had some importance, however, was demonstrated by exposure to *M*/80,000 for 18 hours following cleavage. Here skeleton and slight bilaterality appeared in a few larvae as contrasted to their complete absence when treatment began immediately after fertilization.

5. The effects of zinc chloride when administered with glutathione

Since zinc is known to react with the sulfhydryl groups of certain enzymes it seemed advisable to determine any effects on morphogenesis caused by the addition of a sulfhydryl compound to the experimental solutions of zinc chloride. The present work is preliminary and incomplete since only one sulfhydryl compound (glutathione) was used and all possible experimental variations were not investigated. One questionable procedure in these experiments was the addition of glutathione to sea water (which possibly led to its partial oxidation) but since the eggs of *Dendraster* develop in a sea water medium there seemed to be no other alternative.

Newly fertilized eggs, and in some cases six-hour blastulae, were placed in the following sea water solutions:

1. Sea water (control)
2. 0.1 per cent glutathione
3. 0.05 per cent glutathione
4. O.025 per cent glutathione
5. *M*/40,000 zinc chloride
6. 50 cc. *M*/20,000 zinc chloride plus 50 cc. 0.05 per cent glutathione
7. *M*/80,000 zinc chloride
8. 50 cc. *M*/40,000 zinc chloride plus 50 cc. 0.05 per cent glutathione

It was found that a sea water solution of 0.1 per cent glutathione (sol. 2) did not permit cleavage beyond the two-cell stage and commonly prevented it altogether. With solution 3 development occurred and stunted plutei differentiated (as Fig. 14). Of these, approximately five per cent had evaginated entoderms. Blastulae placed in solution 3 also gave inhibited plutei. Eggs and blastulae exposed continuously to solution 4 (0.025 per cent glutathione) differentiated into plutei that were very close to the control (Fig. 5). A few, however, showed slight inhibition of the oral lobe.

Eggs exposed to solution 5 ($M/40{,}000$ zinc chloride) developed, as previously described, into radial larvae, with basal ciliated bands (Figs. 8–11). Of these, approximately 50 per cent had apical extensions and 10 per cent had exogastrulated. In solution 6, normal plutei, that could not be distinguished from the control, developed even though the concentration of zinc was identical with that of solution 5. Similar results were obtained with solutions 7 and 8 and in experiments starting with the blastula as well as with the one-cell stage. These experiments were repeated and in every case the concentration of glutathione tested gave complete protection from the above concentrations of zinc.

Discussion

These experiments on the modification of developmental patterns in the sand dollar with zinc chloride have shown several interesting things. The cleavage period (first six hours) was not particularly susceptible to this agent except in the higher concentrations. When inhibition did occur it was general. That is, the embryos were inhibited proportionately and developed into stunted bilateral plutei with short anal arms and poorly developed oral lobes. It would seem from these observations that certain processes (enzymatic and otherwise) that are concerned with later differentiations are not very active during the cleavage stage and are therefore less susceptible to zinc. This is in line with the work of Gustafson and Hasselberg (1951) which showed that in the sea urchin embryo the activity of a number of enzymes was constant up to the mesenchyme blastula stage and then increased.

That treatment during cleavage to concentrations of zinc, which do not affect rate, does have some effect on morphogenesis is shown by a comparison of the different experiments. When exposure was begun immediately after fertilization and continued for 24 to 48 hours the embryos showed a higher percentage of exogastrulae and failed to develop stomodaea and skeleton as readily as those in which exposure to zinc was begun after cleavage was completed. Such experiments show that while the larva may recover from the effects of lower concentrations when administered only during the cleavage stage, the inhibition may persist and enhance the effects of exposure after cleavage.

The second six-hour period of development is considerably more sensitive to zinc chloride than is the first six-hour period. Also, at this stage the effects are differential; that is, some processes are inhibited greatly while others are inhibited little if any. This is particularly shown in the development of radial symmetry and in polar elongation. At first glance it would appear that radial symmetry is due to the inhibition of bilaterality but this does not seem to be the case. Rather it appears to be a phenomenon of ventralization (see Rulon, 1949) brought about by the inhibition of dorsal and the spread of ventral regions. In normal development, Child (1941) has shown a gradient in the indophenol oxidase (cytochrome oxidase) reaction which is high at the ventral side but low at the dorsal. It would seem that zinc chloride has less effect on cytochrome oxidase activity than on other activity (perhaps largely enzymatic) located toward the dorsal side. As a consequence the ventral area spreads until it encircles the embryo. Evidence for this is in the radial plutei, which are completely ventralized, with gradations through plutei with unusually large ventral areas and are only partially ventralized.

Polar elongation also seems to be related to gradient differences but in this case those extending from apical to basal regions. Here, again, Child (1941) has shown a differential in indophenol oxidase with the highest activity being at the animal pole. If it is assumed that zinc chloride has less inhibitory effect on enzymatic activity near the apical than near the basal area it may easily be seen how the apical region grows at the expense of basal regions and the embryo elongates in the polar direction. With such elongation the correlating influences between the two regions become farther and farther removed from each other and differentiation of the separated regions becomes more and more independent. In weak solutions, and in embryos removed to sea water, conditioning and recovery phenomena become more apparent with the formation of differentiated apical lobes, apical tufts, stomodaea, etc.

The general effects of zinc chloride are very similar to those of sodium selenite (Rulon, 1952) and cobaltous chloride (Rulon, 1953a) although zinc is effective in approximately one-tenth the concentration required by cobalt or selenium. The effects of these three agents are very different from those of such agents as sodium azide (Rulon, 1950) which strongly inhibit the activity of cytochrome oxidase. Agents which do inhibit this important respiratory enzyme also profoundly inhibit apical development, cause a decrease in size of the ventral area and commonly induce evagination with progressive entodermization.

Zinc, cobalt and selenium have been shown to be inhibitors of sulfhydryl (Potter and Dubois, 1943; Marston, 1952, and others)—in fact, this seems to be one of the few things these ions have in common. A number of enzymes (including succinic dehydrogenase) seems to require the sulfhydryl radical for action (Sumner and Somers, 1953). Cytochrome oxidase does not. In the present work experiments were conducted which showed that eggs and blastulae would develop into normal plutei in solutions of *M*/40,000 and *M*/80,000 zinc chloride if the solutions also had a concentration of 0.025 per cent glutathione. Whether this protection was through reaction of glutathione with zinc inside the cell, at the cell surface, or in the solution outside the cell was not determined. These experiments did not show that zinc was effective in modifying morphogenetic pattern by reacting only with thiol groups in the cell but they may indicate that such a reaction was likely and that at least part of the effect of zinc in these experiments may have been through the inhibition of sulfhydryl radicals. Preliminary experiments (unpublished) have shown that the eggs and young larvae of the sand dollar may similarly be protected from the effects of cobalt and selenium by the addition of glutathione to the test solutions.

Summary

1. Newly fertilized eggs and 6-hour blastulae were exposed to sea water solutions of zinc chloride (*M*/10,000–*M*/320,000) for varying intervals of time.

2. It was found that high concentrations inhibited cleavage while low concentrations caused marked changes in the developmental pattern without much effect in cleavage. The continuous exposure of newly fertilized eggs to strong solutions following fertilization resulted in radial, poorly-differentiated larvae but as the strength of the solution decreased the larvae became elongated in the polar direction and differentiated basal ciliated bands. In the weaker solutions, plutei with large

ventral areas differentiated. In $M/10{,}000$, exogastrulation occurred in 10–25 per cent of the larvae while the differentiation of skeleton was prevented in all concentrations above $M/80{,}000$.

3. Continuous treatment after cleavage gave larvae that were but slightly different from those treated continuously from fertilization. The chief differences were that fewer exogastrulae developed, skeleton differentiated in slightly higher concentrations, and stomodaea developed in solutions that had previously been inhibitory(when treatment began with the one-cell stage).

4. Exposure to high and intermediate concentrations for the first 24 hours following fertilization (followed by return to sea water) caused the larvae to show radial symmetry, polar elongation, and the differentiation of apical and basal lobes. Six hours exposure immediately after cleavage was highly effective in causing the development of radial symmetry and polar elongation, as contrasted to exposure during the six-hour period of cleavage which was relatively ineffective.

5. Developing eggs may be protected from the effects of zinc chloride by adding glutathione to the test solutions. Since the effects of zinc are very similar to those of cobalt and selenium it is suggested that part of the effect of zinc in these experiments may be through the inhibition of enzymes with sulfhydryl radicals.

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MICRO GASOMETRIC DETERMINATION OF DISSOLVED OXYGEN AND NITROGEN[1]

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For polluted water and many biological fluids the Winkler method for the determination of dissolved oxygen may easily become unreliable, or inapplicable. This difficulty can be avoided by using gasometric methods. We describe below such a method for the determination of oxygen (and nitrogen) in one cubic centimeter of water. It has been used extensively under field conditions to analyze, for instance, mud water in mangrove swamps (Scholander, Van Dam and Scholander, 1955), the sap in grape vines (Scholander, Love and Kanwisher, 1955), and the gases in icebergs (Scholander, Kanwisher and Nutt, 1955). The principle and apparatus are based on a micro method for the determination of carbon monoxide in blood (Scholander and Roughton, 1942).

Principle

The water sample, followed by acid, is drawn anaerobically into an extraction syringe pipette, containing bicarbonate in the dead space. The dissolved gases escape into the large CO_2 gas phase generated by the reagents. The liquids are ejected, and the CO_2 is absorbed by strong KOH, leaving the extracted oxygen and nitrogen as a bubble which is moved into the capillary of the extractor. The gas bubble is transferred to a syringe analyzer, where the oxygen is absorbed.

Apparatus

The apparatus consists of two parts: (1) combined water sampler and gas extractor, and (2) gas analyzer.

The extractor (Fig. 1B) consists of a 5-cc. syringe, with a fused capillary tip 5 cm. long, and of 0.7 mm. bore. It is provided with a releasable spring drag, made by passing a wire through a rubber cuff. The syringe is mounted on a wood or plastic base provided with a levelling screw, so that the tip of the capillary can be set to match the cup on the gas analyzer. The capillary tip can be closed with a rubber stopper which has a small hole slit at one end, and can also be provided with a glass cup for KOH (Fig. 1, D and E).

The gas analyzer (Fig. 1A) consists of a millimeter-graduated 0.6-mm. pre-

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cision bore capillary, fused to a $\frac{3}{16}$-inch precision bore glass tubing, which acts as a barrel for a stainless steel plunger, terminating in an $\frac{8}{40}$ screw. The screw is engaged by a spring-loaded lever arm. The analyzer is temperature-stabilized by a plastic water jacket, fluted along the edges and resting on legs to keep it insulated from direct contact with hands and table.

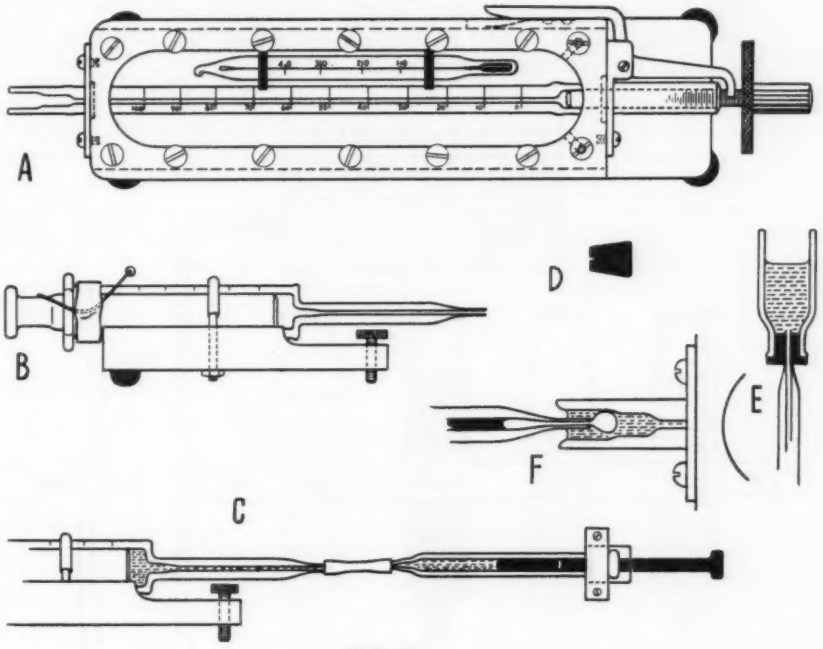

FIG. I

FIGURE 1. A, gas analyzer. B, extractor. C, transfer of water sample from syringe burette to extractor. D, rubber stopper for closing of extractor. E, KOH cup placed on extractor tip. F, transfer of extracted bubble to cup of gas analyzer.

REAGENTS

A. For extracting gases from sample

1. Carbonate solution: Dissolve 8 g. sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$ and 5 g. K_2CO_3 in 20 cc. water.
2. Acid solution: Dissolve 10 g. citric acid and 3 g. sodium citrate in 20 cc. water.
3. Strong KOH solution: Add 100 g. KOH to 60 cc. water in a beaker.

Store (1) and (2) in Erlenmeyer flasks.

B. For gas analysis

1. Acid citrate: Dissolve 170 g. sodium citrate and 6 g. citric acid in 200 cc. water.
2. Alkaline citrate: Dissolve 70 g. sodium citrate and 5 g. KOH in 120 cc. water.
3. Oxygen absorber: Add 15 g. pyrogallol to 100 cc. 20% NaOH in a rubber-stoppered bottle and cover with a layer of paraffin oil 2 cm. thick. The pyrogallol is dissolved under the oil by stirring with a glass rod.
4. Acid rinsing solution: Add 1 cc. concentrated sulfuric acid to 500 cc. water and add 10 mg. potassium permanganate.

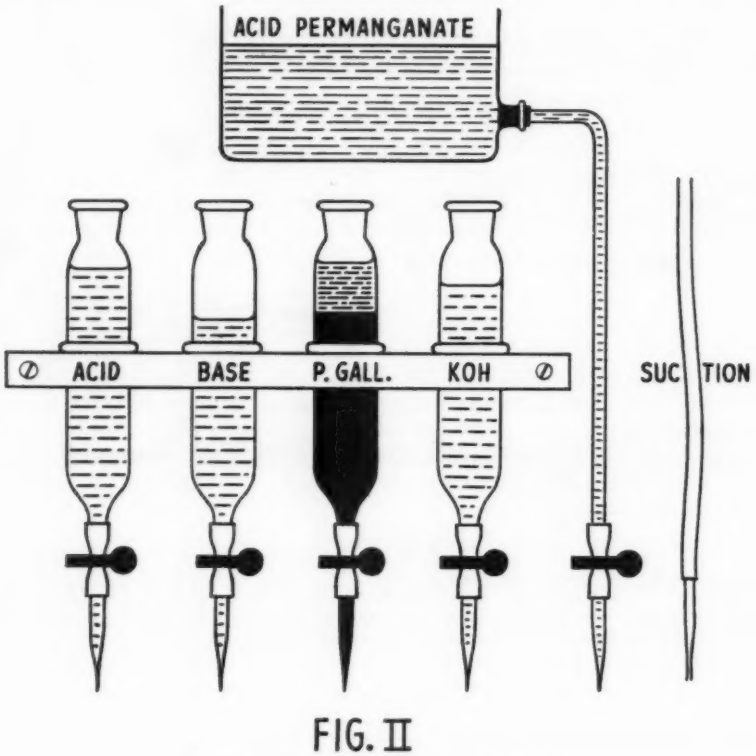

FIGURE 2. Rack for reagents and suction.

The reagents are stored as shown in Figure 2. Solutions A1 and A2 are highly concentrated in order to keep the gas solubility low. The salts crystallize out when cold, so it is often necessary to redissolve them by heating. The solutions are boiled when made up, to overcome the gas supersaturation that results from dissolving the salts.

Calibration

The capillary of the gas analyzer is most easily calibrated by means of a micrometer burette (Scholander, 1942a), with the tip above the bulb running horizontally. With the analyzer mounted horizontally on a stand, and the screw plunger removed, a stiff rubber connection is made between the water-filled cup of the capillary and the tip of the burette, being careful not to trap any air bubbles in the connection. The water is *slowly* and *evenly* moved back in the capillary by means of the micrometer, reading off partial as well as total calibration.

For accurate work it is best to draw the sample into an accurately calibrated syringe burette, with or without automatic stop, as in Figure 1C (Scholander, 1947), and from there deliver it into the extractor syringe. In many cases it will, however, suffice to measure the water sample directly into the extractor, which has been calibrated by delivering water from the one-cc. mark until the plunger touches bottom. During this operation the number on the plunger is kept facing up. The water is delivered into a cylindrical shell vial, about 1 × 3 cm., silicon-coated on the inside. The vial is handled only by forceps. The tall sides and absence of thermal convection effectively prevent evaporation loss during weighing (Scholander, Edwards and Irving, 1943). For repeated deliveries remove the water by means of suction.

Procedure

Collecting sample and extraction of gas

Pour the boiled acid and alkaline solutions (A1 and A2) into two 10-cc. beakers, which are kept covered. Rinse the extractor with water. Draw in ½ cc. of carbonate solution, discard this, and fill the dead space with the same, orienting the plunger with the number up. Either draw in the one-cc. water sample directly or push it in from the burette (Fig. 1C) through a short piece of rubber or plastic tubing. A long and thin-walled polyethylene tube is seriously pervious to gases and should not be used. Trapping of air bubbles must, of course, be scrupulously avoided. The extractor is now held vertically with the tip in the acid, and 0.2 cc. is carefully drawn in. This layers under the carbonate solution, and there is time to close the capillary tip with the rubber stopper before the CO_2 evolution begins. The loss of a few cubic millimeters of gas or liquid at this point is immaterial. With the plunger clip released, the extractor is shaken until CO_2 evolution stops, which with the proper strength of carbonate solution (A1) should be at a gas phase of about 4 cc. Shaking should be continued for two minutes, which is conveniently done by placing the extractor on a rotator. Holding the extractor tip down, the plunger is forced in to give a slight over pressure. The stopper is removed and the extracted water is allowed to run down into a finger bowl of water. Keeping the tip under water as a safety measure all of the syringe fluid is disposed of, leaving only a seal in the capillary. Turn the extractor tip up, readjusting the plunger so as to maintain the liquid seal in the capillary, and attach the KOH cup (Fig. 1E). Fill this half way up with strong KOH (A3). Expel into the KOH any air trapped in the capillary. Draw down the KOH over the CO_2 in the barrel, letting the plunger in as the CO_2 is absorbed. At the very last stage of the absorption, run the remaining bubble of oxygen and nitrogen up into the capillary, where it is safely protected from diffusion exchange until it is transferred to the analyzer.

Transfer and analysis of gas bubble

The analyzer is rinsed and the dead space plus the cup is filled with the alkaline citrate solution (B2). With the extractor and analyzer placed on the table, the extractor tip is introduced into the citrate which fills the cup of the analyzer, and the gas bubble is carefully transferred into the cup by gently twisting the extractor plunger in (Fig. 1F). The analyzer is held cup down, and the gas bubble is drawn into the capillary. With the cup up, the bubble is drawn down into the barrel, and then brought back very *slowly and evenly* into the capillary for a reading. Any abrupt movement of the meniscus leaves excess liquid on the sides of the capillary, and the volume of the bubble will be estimated too large. Enough of the citrate above the gas bubble is drawn off so that both menisci of the seal remain within the capillary during the reading. If the upper meniscus is in the cup, the gas volume reads significantly too large. The readings are made with a hand lens and with the analyzer resting on the table. The length of the bubble is checked by repeating the procedure, and the smallest reading is used (V_1). Oxygen is absorbed by slowly and steadily drawing one cupful of pyrogallol down over the bubble, with the instrument held cup up. The pyrogallol layers around the bubble in the barrel on top of the citrate. By this procedure fresh pyrogallol, in excess, runs over the bubble, which effectively prevents the formation of monoxide. The remaining nitrogen [2] bubble is slowly moved up into the capillary, and the volume is read with all three menisci in the capillary (V_2). Read the temperature of the analyzer.

After analysis, rinse the analyzer several times in water under the faucet, disconnecting the screw feed and removing the plunger. If the water supply is limited, rinsing with permanganate solution (B4) until this retains its color may be necessary.

Calculations

The oxygen contained in the sample equals $(V_1 - V_2 - C)\ (F_s \times i) \times F_a$, where C is the blank correction for oxygen in the reagents, F_s is the factor reducing measured gas volumes to standard conditions (STPD), i is the factor for gas remaining in the liquid phase (near to 1.0075 when liquid volume/gas volume is ¼), and F_a is the calibration of the analyzer capillary. F_s is based on (1) a temperature corrected barometer reading, from which is subtracted the water vapor tension at the analyzer temperature, and (2) the temperature of the analyzer (Handbook of Chemistry and Physics, 1952, pp. 2130 ff. and 1910 ff.).

The nitrogen contained in the sample equals $(V_2 - C)\ (F_s \times i) \times F_a$, where C is the blank correction for nitrogen in the reagents, F_s is the same as above, i is near to 1.004, and F_a is the same as above.

When the analysis is performed at a temperature between 15° and 30° C. the following empirical terms can be used, with less than 0.2% error, as substitutes for $(F_s \times i)$, namely, for oxygen $F_s \times i = 0.215\ B/t + 160$, and for nitrogen $F_s \times i = 0.214\ B/t + 160$, where B is the uncorrected barometric pressure in mm. Hg and t is the temperature of the analyzer in centigrade degrees.

[2] "Nitrogen" here includes the noble gases and other non-absorbable components, like methane, if such are present.

Determination of Reagent Blank C

Half a liter of water is boiled in an Erlenmeyer flask, provided with a cork through which a capillary glass siphon fits loosely. The free end of the siphon has a short piece of rubber tubing with a pinchcock. After half an hour of steady boiling, 10 cc. of the still boiling water are transferred anaerobically through the siphon to a 10-cc. syringe. This is closed with a fine bore rubber tube and glass plug and is cooled under the faucet. The gas-free water is quickly transferred from this into the extractor by means of a short and heavy-walled tubing. Air bubbles must be scrupulously avoided. The extraction and analysis proceed as described. Our blanks amount to 0.8 mm. for oxygen and 2.4 mm. for nitrogen.

Checks on Air-Equilibrated Water

Fifty cc. distilled water are rotated in a 500-cc. open bottle which is kept partly submerged in a water bath of room temperature. Bubbling of air through water results in an unknown degree of supersaturation and cannot be used in this connection. After a half hour's equilibration samples may be taken out. The dissolved oxygen in the sample equals

$$\frac{\alpha \times (B - b - w)}{760} \times 0.2094,$$

where α is the solubility coefficient, B is the read barometric pressure, b the temperature correction for the barometric reading, w the water vapor tension at the water bath temperature, and 0.2094 the oxygen fraction in the air.

Accuracy

The method has been tested on air-equilibrated water as described above. Out of 21 consecutive oxygen analyses 16 were within $\pm 1\%$ of the theoretical value, 4 were within $\pm 2\%$. The average for 20 determinations was 100.1%.

In the same series, 17 nitrogen determinations were within $\pm 1\%$ of the value given in the Handbook of Chemistry and Physics (1952), and 3 were within $+ 2\%$. The average of 20 determinations was 100.6%.

Use of the Instrument for Straight Gas Analysis

For simple gas analysis the analyzer is charged with the acid citrate solution (B1), and a gas bubble is deposited in the cup, using a syringe technique as described by Scholander, Claff, Teng and Walters (1951), or a mercury pipette (Scholander, 1942b). Thanks to the smooth screw control and the water jacket, an accuracy of $\pm 0.2\%$ or better can easily be obtained for CO_2, O_2, and nitrogen. At high carbon dioxide concentrations (20% or more) a slight loss of CO_2 must be expected, unless the sample is drawn into a mercury pipette and delivered into mercury which fills the analyzer cup (cp. Scholander, 1942b).

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